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Editors

Mechanosensitivity in Cells and Tissues 2

Mechanosensitivity of the Nervous System

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Mechanosensitivity in Cells and Tissues

Volume 2

Series Editors

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Mechanosensitivity of the Nervous System

Forewords by Nektarios Tavernarakis and Pontus Persson

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Foreword

Mechanotransduction, the conversion of a mechanical stimulus into a biological response constitutes the basis for a plethora of fundamental biological processes such as the senses of touch, balance and hearing and contributes critically to development and homeostasis in all organisms. Perception of incident mechanical stimuli is critically important for interfacing with the physical world. Naturally, the mechanisms underlying the capability of living cells to receive and act in response to mechanical inputs are among the most ancient, implemented during evolution. Proteins with mechanosensitive properties are ubiquitously present in eubacteria, archaea and eukarya, and are postulated to have been an essential part of the physiology of the Last Universal Ancestor. The first mechanosensitive processes may have evolved as backup mechanisms for cell protection, e.g. to reduce intracellular pressure and membrane tension during osmotic swelling. Subsequent organismal diversification and specialization resulted in variable requirements for mechanotransduction in different organisms. Hence, evolutionary pressure has shaped a large repertoire of mechanotransducers, optimized for a great assortment of tasks that range from maintenance of intracellular osmotic balance and pressure to our impressive ability of hearing and discriminating sounds, and reading Braille code with our fingertips.

Elegant genetic and electrophysiological studies have shown that specialized macromolecular complexes, encompassing mechanically gated ion channels, play a central role in the transformation of mechanical forces into a cellular signal, which takes place in mechanosensory organs of diverse organisms. These complexes are highly efficient sensors, closely entangled with their surrounding environment. Such association appears essential for proper channel gating, and provides proximity of the mechanosensory apparatus to the source of triggering mechanical energy. In addition to the core channel proteins, several other potentially interacting molecules have in some cases been identified, which are likely parts of the mechanotransducing apparatus. Based on cumulative data, a model of the sensory mechanotransducer has emerged that encompasses our current understanding of the process and fulfills the structural requirements dictated by its dedicated function. It remains to be seen how general this model is, and whether it will withstand the impiteous test of time.

Mechanotransduction in living organisms can operationally be categorized as sensory or regulatory. Sensory mechanotransduction or mechanosensation alerts the organism to mechanical inputs in the form of touch, pressure, stretch, sound, vibration and acceleration. Such stimuli provide vital awareness of the environment, and information with regard to the organism's relative position and movement. This prowess is important in negotiating with the physical world and is based on highly adapted mechanotransducers that have evolved to optimally carry out the task.

Both cellular and organismal homeostasis often requires adjustment to mechanical forces generated by environmental sources or internal processes. For example, osmotic balance, ion concentration homeostasis, cell volume and shape regulation, blood pressure and turgor control all depend on appropriately responding to mechanical stretch or shearing forces. Dedicated mechanotransducers in these paradigms serve as regulatory valves that initiate a cascade of events towards adjusting to or counteracting any substantial deviation from normal conditions. The requirement for regulatory mechanotransduction is probably as ancient as life itself. Cells constantly need to fight shearing and stretch forces they encounter, and the faculty of mechanotransduction was most likely decisive for the survival of the first cell. The universal occurrence of mechanotransduction capabilities in all living organisms argues for such early emergence of mechanotransducers.

Sensory and regulatory mechanotransducers obey similar principles and it is likely that the first derived from the second by refinement towards acquiring dedicated functions. In higher organisms, specific neurons, the mechanoreceptors are equipped with a mechanotransducing apparatus and signal upon reception of a stimulus. Frequently, these cells are implanted within accessory structures that serve to filter and amplify an incoming stimulus. For example skin touch receptor neurons are occasionally associated with hair shafts, while hair cells of the inner ear are enclosed in elaborate anatomical structures that greatly facilitate capture and tunneling of sound wave energy.

This book, edited by Andre Kamkin and Irina Kiseleva, provides an excellent point of reference for the current state of the art in the field of mechanotransduction, encompassing authoritative essays on a series of diverse, relevant topics. The articles are properly organized into four parts, with the first part focusing on the mechanosensitivity of nerve cells, the second on mechanoreceptors, the third on the biomechanics of the nervous system and the fourth on the mechanosensitivity of the neurovascular system. Thus, the book provides much needed coverage on key themes of modern mechanotransduction research and is a timely undertaking, which nicely complements the current body of the literature in the field.

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Foreword

Mechanosensitivity of the Nervous System is a unique collection of reviews outlining the current knowledge and the future developments in this rapidly growing field. Mechanosensitivity belongs to the five senses defined in antic times by Aristotle. Mechanosensation is crucial for maintaining life and goes very far back into evolution. Thus, it does not surprise that mechanosensitivity is observed on many levels of the organism. Although it is such an important feature, our knowledge regarding the fundamental principles of mechanosensation remains incomplete. The following contributions of this book shed light onto very different features of mechanosensitivity in the nervous system.

A truly international team of experts have put together their expertises to form this book which is an important step ahead. In the first chapter regarding mechanosensitive channels of the two classes present in the central nervous system, neurons and glia, the importance of mechanosensitive K^+ channels and non-selective cation channels is presented. An in-depth view of transient receptor potential channels (TRP) is then outlined with particular emphasis on the central nervous system. Moreover, the two pore domain potassium channels and amiloride sensitive sodium channels are presented. These latter channels are very important for nociception and, of course, for sodium homeostasis, and it is now becoming increasingly clear that these channels may also play a role for blood pressure control.

Of particular interest is also the highlighting of hormone secretion, as induced by cell swelling. This is very important for osmotic regulation and is thus a crucial mechanism for water and salt regulation. Not only is mechanosensitivity important for the brain and hormone secretion, it is also playing a role in the control of our gastrointestinal tract. Here we find intrinsic neuronal mechano-transductive elements that play an enormous role in the adaptation to filling, and is important for peristalsis, as for local control of blood flow.

In Part 2, mechanosensitivity of the receptors, important work is put together regarding sensory organs. These include the inner ear and again here TRP-channels seem to play a very important role. There is an expert summary of the possible involvement of these TRP-channels in auditory mechanotransduction. Also for the eye, mechanosensitivity is important, as the ocular biomechanics guarantee the shape of the eye. Cells and sensory elements

responding to mechanical stimulation have been found in the cornea, sclera, conjunctiva as well as the uveal tract and the lenses.

In Part 3, the biomechanics of the nervous system is highlighted. Here we find integrative approaches to clarify motor control such as on reaching movements. This is rounded of by very important review over the mechanical properties of brain tissue, and is important to understand the process of injury development in the brain. This is extended by a particular focus on the role of the falx and the internal stress strain response of the brain. The step even further into clinical practise is provided by the outlining of exercise in the nervous system. Here it is specified, how training and exercise can enhance motor recovery in patients suffering from spinal cord injury or stroke.

Finally in Part 4, on the mechanosensitivity of neurovascular system, we again encounter TRP-channels and their function for the regulation of the blood brain barrier.

Taken together this is a truly interdisciplinary summery of our current knowledge regarding mechanosensitivity on nearly all levels of physiology. It spans from insight into the molecular transduction process to the importance in various clinical settings. It is a pleasure to have read this unique contribution and I look forward to the impact this series will have for future science.

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Contents

Editorial

Mechanosensitivity of the Nervous System: Nervous Cells, Receptors, Biomechanic of Tissue

Andre Kamkin and Irina Kiseleva

Mechanosensitivity, i.e. the specific response to mechanical stimulation, is common to a wide variety of cells in many different organisms ranging from bacteria to mammals (see for revives Hamill, 2006; Hamill and Martinac, 2001; Sachs and Morris, 1998). Usually the deformation of single cells and tissue is described in terms of stretch, compression, stress, strain, sheer, bending, twisting, etc. Functional responses to those types of stretch influence different physiological processes, which allow investigator to reveal the link between the functioning of single mechanosensitive ion channels (MSCs) and regulation of particular cellular or tissue functions. In this respect mechanical stress modulates physiological processes at the molecular, cellular, and systemic level.

Nervous system stands out from a number of tissues because besides reacting to the mechanical stress it is transmitting its own response to other organs and tissues, which are located down stream of its signaling pathway. For this reason any of abovementioned types of mechanical stimulation of nervous system, which is capable of triggering it physiological responses, allows to use it beyond a particular experimental model, since it is usually contributing to some particular pathological condition. Currently, investigations of the effects of mechanical stress on nervous system are focused on several issues. The majority of studies investigate the effects of mechanical stimulation on MSCs, as its primary target and interactive agent, and aim on description of downstream intracellular signaling pathways together with addressing general issues of biomechanics of the nervous system. Knowledge of biomechanics, and mechanisms which underlie it on organism, organ, tissue and cellular level, is necessary for understanding of the normal functioning of living organisms and allows to predict changes, which arise due to alterations of their environment, and possibly will allow to develop new methods of artificial intervention.

Present Volume is devoted to discussion of the latest data, obtained in the field of nervous system research. The collection of articles was compiled by a group of international scientists, which are considered to be the leaders in the field of mechanosensitivity of the nervous system. The book comprises four parts, the first of which is discussing mechanically gated mechanosensitive channels of different cells of nervous system, second addressing mechanosensitivity of the receptors, the third describing current vision of biomechanics of nervous system,

and fourth putting together a general vision of mechanosensitivity of neurovascular system. At the same time such set of reviews is rather conventional and is mainly aimed at the reader's convenience since all the presented materials intersect.

For the first time sensitivity to the mechanical stretch of neuronal membrane was studied, when B. Katz described the electrical transduction of membrane stretch in the muscle spindle of frog (Katz, 1950). First investigations in this field addressed issues of mechanosensitivity of Na^+-selective and K^+-selective permeability (Katsuki and Hashimoto, 1969; Thurm, 1970). However the first attempts to study ion channels based mechanisms of mechanosensitivity of the nervous system date back to the beginning of 1980s. During last 40 years there have been a number of interesting studies worth noting. Creation of the patch clamp method allowed Guharay and Sachs to perform their first recordings of mechanosensitive currents in 1984 (Guharay and Sachs, 1984). It has been show that the primary target for mechanical stimulation is the plasma membrane of the cell, which can respond to variable physical stress with changes of the open probability of MSCs. MSCs were shown to convert mechanical force, exerted on the cell membrane, into electrical signals. Since then cellular signaling in response to mechanical stress has been shown to start rapid induction of immediate-early genes, that are transcription factors, and long-term changes in gene expression.

The presence of MSCs in cells of the nervous system originates from the need to maintain general cellular functions, such as control of volume and electrolyte homeostasis and cell movement regulation. In particular, extending neurites of developing or regenerating neurons, which migrate through complex environments to reach appropriate target regions, and the guidance of nerve fibers to their final destination, can be considered as a series of short-range projections under the influence of local cues. Mechanical interaction between cell and substrate is quite important since the nature of the substrate on which growth cones navigate has a powerful effect on the remodeling of the nerve cell processes (Grumbacher-Reinert, 1989). Since adhesion and advance of nerve growth cones are associated both with changes in membrane tension (Lamoureux et al., 1989), and with transient elevations of intracellular calcium ($[Ca^{2+}]_i$) (Gomez and Spitzer, 1999), MSCs play a critical role in the mechanotransduction response (Gomez et al., 1995; Ingber et al., 1990). To better understand the mechanisms by which cells transduce changes in membrane tension into different biochemical responses, which regulate growth and differentiation, we should know how environmental factors affect MSCs and how signals, generated by MSCs, are integrated with other messengers inside the cell (Pellegrino et al., 2005).

For this reason, the first part of our book contains description of studies, dealing with MSCs, and this part of the volume is opened with an article devoted to MSCs, which are expressed in the plasma membrane of both non-sensory neurons and glial cells, which make up the major non-neural cellular component of the CNS and are responsible for a number of important functions

(Kirischuk, 2008). Astrocytes, which express MSCs, serve as a representative example of such cells. Two pore domain potassium channel family, three of which $K_{2P}2.1$ (TREK-1), $K_{2P}10.1$(TREK-2), $K_{2P}4.1$ (TRAAK) can be activated by mechanical stress, are discussed along with mechanosensitivity of other ion channels. For example, the non-selective cation channels of the vanilloid subfamily (TRPV) are reported to be implicated in sensing and transduction mechanisms of osmotic stimuli. After that Volume describes MSCs, which are expressed in neurons. We think that findings of the investigation of synaptic release, to which MSCs significantly contribute, can be of particular interest to our readers together with discussion of the role of MSCs in functioning of neuro-glial functional networks (Kirischuk, 2008).

The following review addresses MSCs, expressed by neurons in detail (Chung and Oh, 2008). It contains a throughout description of the development of our vision of mechanosensitivity of neurons and defines the criteria, which is used to determine the mechanical gating of ion channels. In this chapter authors describe transient receptor potential non-selective cation channel superfamily, two pore domain potassium channel family, amiloride sensitive sodium channel family and voltage-gated ion channels. Authors consider transient receptor potential, otherwise referred as TRP channels, from the point of view of the relationship between their structure and function with that of six other ion cannel families: TRPC (Canonical), TRPM (Melastatin), TRPV (Vanilloid), TRPA (Ankyrin), TRPP (Polycystin) and TRPML (Mucolipin), based on amino-acid homologies. Here readers will also find a detailed description of two pore domain potassium channel family, with a focus on $K_{2P}2.1$ (TREK-1), $K_{2P}10.1$(TREK-2), $K_{2P}4.1$ (TRAAK) channels, and that of Degenerin/Epithelial sodium channels (DEG/ENaC), the story of which began, when Jorgensen and Ohmori (1988) reported that amiloride blocked the hair cell transduction channel of a chick. The overview of different experimental methods, which are used to study MSCs, concludes this part of the Volume (Chung and Oh, 2008).

Next review is devoted to mechanosensitive cation currents and their molecular counterparts in mammalian sensory neurons (Hao et al., 2008). Mechanoreceptive somatosensory neurons are responsible for the transduction of mechanical stimuli into action potentials that propagates to the central nervous system. The ability of these sensory neurons to detect mechanical stress relies on the presence of MSCs. For the first instance authors outline methodological approaches of studying MSCs, considering several types of mechanical stimuli, which have been used to investigate MS channels, including piezo-driven pressure, patch membrane stretch, shear stress, osmotic challenges and amphipathic compounds. After that authors proceed to discussion of biophysical properties of mechanosensitive currents in sensory neurons. The description of MSCs, which are typical tosensory neurons, concludes this chapter (Hao et al., 2008).

Following review addresses neurons and cell swelling-induced peptide hormone secretion (Štrbák, 2008). Authors discuss how cell swelling, which is induced by extracellular hypotonicity or intracellular hypertonicity, is integrated into a signal transduction network, which is regulating various cell

functions, including apoptosis, and describe how cell volume regulation is linked with peptide secretion and how it induces secretion from different parts of the neuron: axons, dendrites and bodies. This chapter demonstrates how cell swelling-induced exocytosis possesses limited selectivity, how cells, specifically involved in water and salt regulation, retain their specific response to osmotic stimuli; and how neurons of the hypothalamic supraoptic and paraventricular nuclei release oxytocin, vasopressin and angiotensin II and III in response to hyperosmotic stimulation. Authors use particular examples how those specific responses can be altered and discuss clinical relevance of those phenomena's (Štrbák, 2008).

The book goes beyond the discussion of the mechanosensitivity of neurons of central nervous system. Last chapter of the First Part of the Volume is devoted to the neuronal mechanosensitivity in the gastrointestinal tract (Smid, 2008). This review focuses on the neuronal mechanosensitive elements within the gastrointestinal tract and describes their functional and neuroanatomical features. The gastrointestinal tract possesses a substantial network of sensory neural circuitry that conveys information both within the gut and to the central nervous system. Authors describe gastrointestinal mechanosensory pathways and discusse intrinsic neurons, which are a number of cell types believed capable of sensory transduction and which utilise intrinsic sensory nerves in this process, and describe extrinsic neurons. The extrinsic pathways are conveyed via both vagal and spinal nerves. Review includes analysis of pharmacological modulation of mechanosensory nerves and that of the role of mechanosensory nerves in gastrointestinal diseases (Smid, 2008).

The Second Part of the Volume is dealing with mechanosensitivity of the receptors. The first chapter of the Second Part by Tabuchi and Hara (2008) addresses mechanosensitivity of the cochlea. Hair cells are mechanosensitive cells, which are essential for transduction of sound stimuli. Authors outline possible involvement of TRP channels in auditory mechanotransduction and consider the contribution of TRPV (TRPV1, 2, 3 and 4), TRPA1 and TRPML3 channels to it.

As a logical development of the previous review next Chapter analyses osmoreceptors in cochlear outer hair cells (Harada, 2008). It describes how passive calcium-independent slow motility, which is induced by hyposmotic activation, may have physiological or pathological significance even in normal or impaired hearing of cochlear origin. Chapter discusses a recent finding that the cell swelling, which is induced by hypoosmotic stimulation, has been shown to be accompanied by an increase of intracellular Ca^{2+} concentration in mammalian outer hair cells, and the hypothesis that this increase may subsequently activate metabolic processes, including phosphorylation in cells. This manuscript proposes that the functional expression of TRPV4 is involved in the hypotonic stimulation-induced Ca^{2+} influx in mammalian outer hair cells. Authors propose that hyposmotic stimulation can induce nitric oxide production by the $[Ca^{2+}]_i$ increase and analyze in detail the role of nitric oxide (Harada, 2008).

Next review by Tan and Coroneo (2008) brings up the question – "Are stretch-activated channels an ocular barometer?" The ocular biomechanics facilitating vision are orchestrated by a complex interplay between cells and their immediate environment within the tissues of the eye. Cells that respond to mechanical stimulation have been identified in the cornea, sclera, conjunctiva, lens and uveal tract (Belmonte et al., 1997; Cooper et al., 1986; Mintenig et al., 1995). Analyzing published findings authors note that stretch inactivated channels were not identified within the tissues of the eye and that this actually adds to controversy, created by works by Honoré et al. (2006), Sunchya and Sachs (2007), about existence of stretch inactivated and pressure activated channels (for a detailed discussion please see Kamkin and Kiseleva, 2007). In their Chapter Tan and Coroneo (2008) also provide a detailed discussion of effects of pressure on eye cells and of their possible relevance to glaucoma. This topic looks especially interesting from a clinical prospective. This part is concluded by a review by Leitch and Pitman (2008), which describes neuromodulation of mechanosensory input to the insect CNS. The manuscript analyzes presynaptic modulation of sensory information and postsynaptic modulation of mechanosensory input.

The Third Part of the present Volume is dealing with biomechanics of nervous system. This part is very important, because CNS cells are confronted with very special environmental conditions, and because much of their development, functioning, and pathology cannot be properly understood without knowledge of their biomechanics. Similarly to electrophysiological studies, investigations of biomechanics of nervous system began with measurements of mechanical properties of brain tissue in the beginning of 1970s (Fallenstein et al., 1969; Ommaya, 1968; Shuck et al., 1970; Shuck and Advani, 1972). The first Chapter of the Third Part defines the topic of biomechanics of the CNS (Franze et al., 2008). It provides a comprehensive discussion of physical basics, which are necessary to understand biomechanical measurements. Since the cytoskeleton is widely accepted to be the structure, which is responsible for inherent mechanical cellular properties, authors discuss the properties of this network. Chapter contains a detailed description of viscoelastic properties of the CNS and viscoelastic properties of CNS cells (Franze et al., 2008). Following review is dealing with the neural representation of kinematics and dynamics in multiple brain regions. (Francis, 2008). Authors debate what movement related variables are controlled by the neural motor control system, considering dynamic vs. kinematic variables. This Chapter is followed by the review "Mechanical properties of brain tissue: Characterisation and constitutive modeling" (van Dommelen et al., 2008) and a chapter that describes how physical and finite element models have shown that the falx cerebri, which is present in the human head, affects the intrinsic response of the brain under contact- and inertia-induced dynamic loads. Chapter "Exercise and nervous system" concludes Third Part with practical and clinical implications of the mechanosensitivity of nervous system to the general public and clinical practitioners from the point of view of exercise physiology (Imai and Nakajima, 2008).

The Fourth Part of the Volume begins with a Chapter by Brown and O'Neil (2008) Mechanosensitive calcium fluxes in the neurovascular unit: TRP channel regulation of the blood-brain barrier. The blood-brain barrier of the neurovascular unit is a critical organ for normal brain function. This review describes the molecular identity of the channels, which are underlying mechanosensitive calcium influx, and hypothesizes a central role for transient receptor potential channels in mechanosensitive regulation of blood-brain barrier endothelial cell function (Brown and O'Neil, 2008).

The volume dwells on the major issues of mechanical stress, which are influencing the ion channels, intracellular signaling pathways and biomechanics of the nervous system. In our opinion the book brings up the problem closer to the experts in related medical and biological sciences as well as practicing doctors besides just presenting the latest achievements in the field. We hope that presenting the problem will attract more attention to it both from researchers and practitioners and will assist to efficiently introduce it into the practical medicine.

NOTE: Current Volume contains a number of reviews, one of which is dealing with TRPC1 and TRPC6 channels and their mechanosensitivity, which was recently reported in a number of experimental papers. When the Volume was already in print Gottlieb et al. (2008) published their manuscript, which is revising this topic. Authors reported that the amplitude of the mechano-sensitive current is not significantly altered by overexpression of TRPC1 or TRPC6 subunits. After taking into consideration unquestionable reputation of this group (Gottlieb P, Folgering J, Maroto R, Raso A, Wood TG, Kurosky A, Bowman C, Bichet D, Patel A, Sachs F, Martinac B, Hamill OP, Honoré E) in the field of mechanosensitivity we would like to ask our readers to consider issues of TRCP1 and TRCP6 mechanosensitivity in the light of those latest findings.

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Part I
Mechanosensitivity of Nervous Cells

Chapter 1
Mechanosensitive Channels in Neuronal and Astroglial Cells in the Nervous System

Sergei Kirischuk

Abstract The central nervous system consists of two cell classes, neurons and glia. Mechanosensitive channels are expressed on the plasma membrane of both non-sensory neurons and glial cells. Different chemical stimuli, such as neurotransmitters, or poly-unsaturated fatty acids, and stimuli associated with membrane deformation, such as perfusion with hypo-osmotic solutions or local mechanical stress, influence the activity of these channels. Several mechanosensitive K^+, such as TREK/TRAAK family, and non-selective cation channels, such as transient receptor potential family, have been recently cloned, and their expression in both neurons and astroglia has been demonstrated. The main aim of this article is to summarize the available data on mechanosensitivity of these two cell types. Possible roles of recently characterized MS channels in the brain development and functioning are discussed.

Keywords Mechanosensitive channels · astrocytes · neurons · glia

1.1 Introduction

Living organisms are able to sense and respond to environmental changes. Sensory systems are typically coupled to activation of ion channels which convert specific stimuli into electrical signals. These ion channels are either directly activated by the stimulus or indirectly by chemical components of a transduction cascade. Mechanosensitive (MS) channels convert mechanical force exerted on the cell membrane into electrical signals during physiological processes such as touch and hearing in mammals (Vollrath et al., 2007; Corey, 2003; Hamill and McBride, Jr., 1996). Selection mechanisms favor retention of mechanotransduction during evolution (for review (Martinac and Kloda, 2003)), and it is likely that such channels play a fundamental role in cellular physiology. The patch-clamp technique first allowed direct measurement of

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single MS channel currents (McBride, Jr. and Hamill, 1993; Guharay and Sachs, 1984). Despite the fact that the patch-clamp technique used to activate and investigate MS channels led to an interpretation of the observed MS channel activities as artifacts (Morris and Horn, 1991), the cloning and characterization of several MS channel proteins eliminated all uncertainties.

The structural diversity of MS channels appears to come from the physiological necessity to detect pressures ranging by several orders of magnitude. Some detectors, like auditory hair cells, use cytoskeleton-mediated fine-tuning of channel sensitivity. Many types of mechanically-activated currents do not require intact cytoskeleton or extracellular matrix, and are elicited by tension in the lipid bilayer. These channels could implement necessary feedback in tissues that generates large tensions themselves, such as skeletal and heart muscles. Osmotic swelling represents a third type of perturbations leading to activation of MS channels (for review see (Sukharev and Anishkin, 2004; Hamill, 2006; Hamill and Martinac, 2001)). Interestingly, many cell types, including non-specialized neurons, which are not involved in mechanoperception, have been shown to express MS channels. These channels can be related to the general functions of all cells such as volume and electrolyte homeostasis or regulation of cell growth and motility. Indeed, MS channels have been shown to play an important role in cell motility (Sukharev and Anishkin, 2004; Lamoureux et al., 1989), and embryogenesis (Bregestovski et al., 1992).

The understanding of mechanosensation has shown considerable progress in recent years. Genetic screening in model organisms like nematodes, flies, zebrafish and mice have identified several genes encoding channel-like molecules involved in mechanosensory responses, including two-pore-domain K^+ channels and transient receptor potential (TRP) channels. An entire family of genes has been described whose members generate the hallmark properties of leak K^+ channels, namely K^+ selectivity and relative time- and voltage-independence. Genes from this family differ from other K^+ channel genes in their predicted structure, with each subunit containing two pore-forming loops (P domains) instead of one. Therefore, members of this family are referred to as "two-pore-domain" K^+ channels (K2P channels). To date, 14 K2P channel genes from the human genome have been described. Further investigations have shown that three K2P channels (TREK-1, TREK-2 and TRAAK) are mechanosensitive. All of them are expressed in the CNS (Patel et al., 2001; Talley et al., 2001; Hervieu et al., 2001). Moreover, genetic inactivation of TREK-1 in the mouse has revealed its potential involvement in a range of neuronal disease states, including pain, epilepsy and depression (Heurteaux et al., 2006; Alloui et al., 2006; Heurteaux et al., 2004).

The other family of potential mechanoreceptors is the transient receptor potential (TRP) family. TRP channels are one of the largest group of ion channels, but they were only recently uncovered in full through the elucidation of complete genome. In humans, at least 28 genes in six families can be classified as TRP channels. They are weakly voltage-sensitive, largely nonselective, cation channels. With the exception of capsaicin for TRPV1, there are no currently available

pharmacological agents that can be used to separate their physiological function. However, three of TRP channels (TRPC1, TRPV2, TRPV4) can be activated by mechanical stretch and/or osmotic swelling (for review (Venkatachalam and Montell, 2007; Christensen and Corey, 2007; O'Neil and Heller, 2005; Maroto et al., 2005)). Similar to K2P channels, mechanosensitive TRP channels are expressed in the CNS (Moran et al., 2004). In this review we will summarize the findings in the physiology of MS channels in two main types of cells in the brain: astroglial cells and neurons, and the possible role of mechanosensitivity in the brain development and functioning will be discussed.

1.2 Astrocytes

Glial cells make up the major non-neural cellular component of the CNS. Historically, they were considered to play mainly a structural role and even the word "glia" comes from the Greek for "glue". Indeed, first electrophysiological recordings using microelectrodes have not discovered any voltage-gated ion conductance supporting the view of glia as an electrically silent element on the nervous system (Kuffler et al., 1966; Ransom and Goldring, 1973). However, appearance of high-sensitive patch-clamp technique led to a revolution in glial cell electrophysiology, clearly demonstrating that these cells express a variety of ion channels and neurotransmitter receptors (for review see (Verkhratsky and Steinhauser, 2000)). Glial cells, which account for approximately 90% of all neural cells in humans, perform a variety of vital functions: radial glial cells control neurogenesis, neural cell development and migration; oligodendrocytes are responsible for axonal myelination; microglia acts as a main defense system in the CNS; astrocytes are involved in synaptic transmission. Astrocytes are the most abundant cells in the CNS. These cells are coupled via gap junctions and form a three-dimensional network that extends through the brain. Astrocytes sense neurotransmitters and can release physiologically active substances (Kimelberg et al., 1990b; Panatier et al., 2006; Bezzi et al., 2004; Cotrina et al., 1998). Although astrocytes can not generate action potentials, they demonstrate another form of intercellular communication, namely Ca^{2+} waves (for review see (Fiacco and McCarthy, 2006; Verkhratsky et al., 1998)). Ca^{2+} signals propagating through astrocytic syncytium can be elicited by a variety of physiologically active substances. However, the most intriguing observation was that a mechanical stimulation of a single glial cell resulted in a wave of increased Ca^{2+} concentration that was communicated to surrounding cells (Charles et al., 1991). This data shows that astrocytes can sense mechanical stress and translate it into chemical messages.

Astrocytes in situ are known to swell in a variety of pathological states affecting the CNS, like stroke, trauma, and hepatic encephalopathy. Isolated astrocytes have been shown to display regulatory volume decrease whereby after initial swelling the cells decreased their volume back to the control level

during continued exposure to hypotonic medium. Using sharp microelectrodes, Kimelberg and O'Connor (1988) showed that cell swelling was accompanied with a strong (up to 60 mV) membrane depolarization, while a gradual repolarization coincided with the regulated volume decrease. The results of ion replacement experiments demonstrated that the swelling-induced depolarization may be explained on the basis of the opening of Cl^- channels, whereas the slow repolarization during cell shrinkage most probably reflects the opening of K^+ or non-selective cation channels. Indeed, a series of studies confirmed that both anion- (Pasantes-Morales et al., 1994) and cation-permeable (Kimelberg et al., 1990a) channels contribute to the hypo-osmotic medium-induced membrane potential changes.

Although increase in intracellular Cl^- concentration accompanying astroglial depolarization suggests the presents of electrogenic Cl^- transport, first reports of single channels in resting astrocytes indicated that Cl^- channels were normally not functional and their activation required excision of the membrane patch (Barres et al., 1990; Jalonen, 1993). Usage of the whole-cell patch-clamp technique also failed to detect any Cl^- conductance in cultured astrocytes with flat, polygonal morphology. However, several manipulations that alter cell morphology, including trypsin treatment, application of serum-free solution, exposure to hypo-osmotic solution, elicited whole-cell Cl^- currents (Lascola and Kraig, 1996). Further experiments demonstrated that cytoskeletal actin changes play an important role in the induction of Cl^- currents. Substances which inhibit actin polymerization (cytochalasins, DNase) elicited whole-cell Cl^- conductance in flat astrocytes, while drugs which stimulate actin polymerization inhibited Cl^- currents (Lascola and Kraig, 1996; Lascola et al., 1998; Crepel et al., 1998). Interestingly, elevated intracellular Ca^{2+} concentration, acting presumably via actin depolymerization, enhanced Cl^- currents (Lascola and Kraig, 1996). A modulatory role of cytoskeletal actin on the open-state probability of inducible Cl^- conductance has been directly demonstrated at single-channel level (Lascola et al., 1998; Crepel et al., 1998). Taken together these results suggest that (1) an interaction between Cl^- channels and cytoskeleton is involved in astrocyte volume plasticity, and (2) this regulatory mechanism can be modulated by intracellular Ca^{2+}.

Cation-permeable MS channels have been demonstrated on the soma of neonatal rat astrocytes in primary cell cultures using the single channel recording technique. In these studies MS channels were activated by changing pressure in the back of the pipette (Bowman et al., 1992; Islas et al., 1993). The presence of a K^+ selective MS channel was described in cerebellar cultured astrocytes (Islas et al., 1993). The open probability of this channel increased with suction (negative pressure applied to the intra-pipette space) with half activation at 45 mm Hg. In the other study (Bowman et al., 1992), five different MS channels were observed on the soma of neonatal rat cortical astrocytes. Four classes of MS channels were found to be K^+ selective. They displayed a linear current-voltage relationship and were activated by both negative and positive pressure. The fifth class of observed MS channels could be activated only by increasing

the pipette pressure. The authors called this type of MS channels a curvature-sensitive or CS channel. The CS channels showed an inwardly rectifying current-voltage relationship and were characterized as non-selective cation channels. Interestingly, the activity of CS channels was observed in the cell-attached configuration, but not in isolated membrane patches. Based on the observed properties of CS channels, it was suggested that these channels can respond to membrane tension only when membrane geometry is in the permissive configuration, for example astrocytic processes surrounding synapses. Identification of specific antagonist of cationic MS channels GsMTx-4 (for review (Bowman et al., 2007)), a peptide toxin from Grammostola spatulata spider venom, allowed demonstrating MS channel contribution to astrocytic volume regulation. GsMTx-4 selectively blocked MS channels in outside-out patches from adult rat astrocyte and strongly suppressed swelling-activated whole-cell currents (Suchyna et al., 2000).

The above results demonstrate that astrocytes express MS channels. However, they are not fully characterized at the molecular level. Several K^+ channels with two pore-forming domains (K2P) have been recently cloned. Three of them (TREK-1, TREK-2, TRAAK) can be activated by mechanical stress. Functional expression of TREK-2 K^+ channel in cultured brain astrocytes has been demonstrated using RT-PCR and single channel recordings techniques (Gnatenco et al., 2002). Interestingly, TREK-2 activity has been reported to be generally low under resting conditions, but they could be activated by applying mechanical stimulus or polyunsaturated fatty acids (Gnatenco et al., 2002; Ferroni et al., 2003). In addition, spatial distribution of TREK-2 K^+ channels appeared to be non-uniform. Several (2–6) channels have been observed in some membrane patches, whereas no channels were activated in other patches by pressure and/or fatty acids. This data allows suggesting that TREK-2 K^+ channels may be clustered on the membrane of the astrocyte plasma membrane. Definitely, single channel recordings from the soma of cultured cells complicate any physiological interpretation of the obtained data, but it is tempting to speculate that activation of TREK-2 clusters at astrocytic compartments which can experience osmotic perturbations (for example, perisynaptic astrocytic processes) may be of importance for brain functioning.

Another important question is whether an activation of MS channels alone is sufficient to trigger inter-cellular Ca^{2+} waves in astrocytic network. The obtained experimental data shows that (1) astrocytes express several types of MS channels; (2) mechanical stimulation as well as cell swelling (Fischer et al., 1997) results in an increase of intracellular Ca^{2+}, which involves Ca^{2+} influx and release from intracellular Ca^{2+} stores; (3) this local Ca^{2+} signal can propagate from cell to cell, forming an intercellular wave. Actin fiber assembly was shown to be of crucial importance for inter-cellular astrocytic Ca^{2+} signaling. Although neonatal astrocytes exhibit extensive gap-junction coupling shortly after placing in culture, Ca^{2+} waves do not propagate until the definite actin cytoskeleton develops (Ostrow and Sachs, 2005). The radius of

propagated Ca^{2+} waves has been shown to be directly proportional to the fraction of cells with organized stress fibers, and disruption of actin cytoskeleton or inhibition of myosin light chain kinase attenuated Ca^{2+} waves. However, the channel type(s) underlying mechanically-induced Ca^{2+} response in astrocytes remains to be identified at the molecular level. Astrocytes both in cultures and in slice preparations express voltage-operated and ligand-gated Ca^{2+} channels (Duffy and MacVicar, 1994; Kirischuk et al., 1999; Kimelberg et al., 1997). Therefore, a non-mechanical Ca^{2+} influx can be indirectly activated by membrane deformation. For example, the regulated volume decrease observed in astrocytes in hypotonic solution could occur as follows. Hypotonic medium induces water flux that swells the cells and activates MS channels. Ion fluxes through MS channels result in a depolarization of the cells which is followed by opening of voltage-operated Ca^{2+} channels and Ca^{2+} influx into the cells. Intracellular Ca^{2+} activates Ca^{2+}-dependent K^+ and Cl^- channel to reduce the swelling (O'Connor and Kimelberg, 1993).

Although one can not reject the above scenario, there is some evidence that Ca^{2+} influx through MS channels can be responsible for the initial Ca^{2+} elevation. In particular, the non-selective cation channels of the vanilloid subfamily (TRPV) have been reported to be implicated in sensing and transduction mechanisms of osmotic stimuli (for review see (Liedtke, 2006)). Functional TRPV4 channels have been recently demonstrated in rat cortical astrocytes both in cultures and in brain slices (Benfenati et al., 2007). Application of hypo-osmotic saline induced an increase in intracellular Ca^{2+} concentration, and this Ca^{2+} response was blocked by TRPV4 channel antagonists. Interestingly, using immunohistochemistry and immunogold electron microscopy TRPV4 channels were shown to be enriched in astrocytic processes of the superficial layers of the neocortex and in astrocyte end feet contacting the pia surface and enwrapping blood vessels. In vivo studies in TRPV4 knockout mice showed that genetic ablation of this channel led to impaired osmotic sensation and altered volume regulation of the extracellular compartment (Liedtke and Friedman, 2003; Liedtke and Kim, 2005). In vitro studies suggested a critical role of TRPV4 in regulated volume decrease in the astrocytes in response to hypo-osmotic stress (Liu et al., 2006; Becker et al., 2005). As hypotonicity-induced Ca^{2+} increase in astrocytes was inhibited by TRPV4 channel blockers, these channels appear to contribute to the regulated volume decrease phenomenon (Benfenati et al., 2007). Notably, water-channel proteins, aquaporins, are also expressed in astrocyte end feet (for review see (Tait et al., 2008)), which suggests that TRPV4 and aquaporins may act in concert in the regulation of extracellular osmolyte/water homeostasis. In addition, there is evidence that astrocytes play a distinct role in neurovascular coupling. Glutamate released at active synapses triggers Ca^{2+} elevations in astrocytes (Pasti et al., 1997), and these Ca^{2+} oscillations have been shown to propagate to perivascular end feet (Zonta et al., 2003) suggesting that astrocytes can transfer to blood vessel information of the level of neuronal activity. Whether MS channel-(TRPV4-) mediated Ca^{2+} elevations in the astrocyte end feet, induced, for example, by

osmotic stress or arachidonic acid derivatives, can propagate in the opposite direction remains, however, to be demonstrated.

1.3 Neurons

1.3.1 Cell Migration

The migration of postmitotic neurons from their site of origin to their final destination, where they make synaptic connections, is a fundamental cellular event essential for the building of the brain. The migration of neurons requires the orchestration of multiple molecular events, including the formation of adhesive interactions with cellular and extracellular substrates and the assembly and disassembly of cytoskeletal components (Yacubova and Komuro, 2003; Schwab et al., 2007). All migrating cells are polarized morphologically and functionally along the axis of movement. A repeated and coordinated protrusions of the front part and retraction of the rear part of the cell results in directed migration. The cytoskeleton is probably the most important cellular motor for cell migration. The front part comprises a dense meshwork of actin filaments. Their polymerization mediates plasma membrane protrusion at the leading part of a migrating cell. The retraction of the rear part of a migrating cell is mediated among others by the contraction of actomyosin network at this cell pole (for review see (Mogilner and Oster, 2003)).

Thus, coordinated cytoskeleton assembly and disassembly is of crucial importance for cell migration. The integrity of cytoskeleton is strongly dependent on cell volume. Cell swelling leads to actin depolymerization, while cell shrinkage promotes actin polymerization. Ion channels play a pivotal role in cell volume regulation and, on the other hand, cytoskeletal components regulate ion channel activity (Lang et al., 1998; Schwab et al., 2007). Because cell migration is a Ca^{2+}-dependent process (Zheng and Poo, 2007; Komuro and Rakic, 1998), fine tuning of intracellular Ca^{2+} concentration is a prerequisite for correct cell migration. For example, intracellular Ca^{2+} concentration has been shown to be spatially heterogeneous with the lower concentration at the front cell pole (Brundage et al., 1991). Mechanical tension in migrating cells can occur when the rear part is stuck to the substratum, while the front pole is actively protruding. Indeed, lamellipodial extension rate demonstrate an inverse correlation with the mechanical tension (Raucher and Sheetz, 2000). Thus, tension-sensitive K^+ channels, for example the TREK/TRAAK family, and Ca^{2+}-permeable channels, like TRPC1 or TRPV4, may be linked to migration.

Direct involvement of stretch-activated channels in cell migration has been shown keratinocytes, fibroblasts and hepatoblastoma (HepG2) cells (Lee et al., 1999; Munevar et al., 2004; Vriens et al., 2004). Migrations of neuronal cells that migrate postnatally to their final position appeared to be dependent on the

activity of other Ca^{2+} channels (for review see (Komuro and Rakic, 1998)). NMDA receptors and N-type calcium channels have been shown to play important, stage-specific roles in controlling migration of cortical neurons (Behar et al., 1999) and granule cells from the developing cerebellum (Komuro and Rakic, 1993, 1992). Interestingly, both NMDA receptors and N-type Ca^{2+} channels have been shown to be mechanosensitive. A link between NMDA receptors and cytoskeleton have been originally suggested by the data showing that NMDA channel activity can be modulated by the actin polymerization state (Rosenmund and Westbrook, 1993) and, vise versa, NMDA channel activity can modulate cytoskeletal proteins (Halpain and Greengard, 1990). Modulation of NMDA channel activity by osmotic and hydrostatic pressure has been directly demonstrated in cultured cortical mouse neurons (Paoletti and Ascher, 1994). Although mechanosensitivity of N-type Ca^{2+} channels in situ has not been shown yet, stretch-induced increase in peak current and in the open probability of single channel have been demonstrated in transfected HEK cells (Calabrese et al., 2002). One can not exclude that yet unknown MS K^+ and/or non-selective cation channels are involved in neuronal cell migration, but it could also be the case that mechanical modulation of neuronal migration is mediated by mechanosensitive NMDA receptors and N-type Ca^{2+} channels.

1.3.2 Neurite Outgrowth and Growth Cone Motility

Neurons, with distinct axonal and dendritic territories, are polarized cells. Neuronal polarization occurs when one of the multiple neurite emerging from the cell body elongates rapidly. This neurite becomes the axon, whereas the remaining neurites become dendrites (Dotti et al., 1988). Actin assembly and disassembly play an important role in neurite elongation and growth cone movement (Forscher and Smith, 1988; Lin et al., 1994). Moreover, axon formation has been shown to take place from the growth cone with a more dynamic and labile actin network (Bradke and Dotti, 1999). In addition, it has been shown that filipodia of the growth cone create pulling tension needed for neurite elongation (Lamoureux et al., 1989) and neurite elongation rate is a linear function of pulling tension (Zheng et al., 1991). These observations allow suggesting that there is a link between mechanical tension and axonal specification: the neurite that first exerts sufficient tension and begins rapid elongation becomes an axon. Interestingly, when a mechanical force was applied with a towing needle to neurite, the towed neurite developed a growth cone and advanced rapidly becoming an axon (Lamoureux et al., 2002). Thus, mechanical tension seems to be a proximal stimulus that initiates axonal specification, however, it is not shown yet that MS channels are involved in this process.

Neurite membranes definitely experience tension changes during growth, but growth cones of CNS neurons are usually not assessable electrophysiologically.

Therefore, the expression of stretch-activated channels was first studied in growth cones suitable for patch clamp experiments. Growth cones of snail neurons were shown to express functional stretch-activated channels (Sigurdson and Morris, 1989; Belardetti et al., 1986). The observed channels were selective K^+ channels and their activation was steeply dependent on the membrane tension (pressure applied through the recording pipette). As motile growth cones have been shown to have higher Ca^{2+} concentrations than those that have stopped growing (Cohan et al., 1987), MS K^+ channels have been suggested to provide a negative feedback mechanism.

Functional MS K^+ channels observed in the growth cones of developing snail neurons share all the properties of the two-pore-domain K^+ channel TREK-1 (Honore, 2007). Lauritzen et al., (2005) have shown that EYFP-TREK-1 expression in neonatal hippocampal neurons induced a profound change in the morphology of these cells. The relative surface area of neurons expressing TREK-1 was almost two times larger than that of TREK-1-negative cells. Numerous filopodia-like structures were observed in the dendritic tree and the axon, and, moreover, strong co-localization of TREK-1 and actin was observed in these protrusions. Comparing the morphology of cultured striatal neurons, which naturally express high levels of TREK-1, with that of TREK-1 knockout mice revealed that genetic ablation of TREK-1 reduced the proportion of expanded growth cones (Lauritzen et al., 2005) suggesting that TREK-1 channels play an important role during early neuronal development. Interestingly, the morphologic effects of TREK-1 seem to be inversely related to the channel activity. Under control conditions, TREK-1 is almost never active due to a tonic repression by actin cytoskeleton, whereas mutations that increase TREK-1 channel activity have been shown to strongly suppress its morphological effects.

Thus, TREK-1 seems to provide a negative feedback suppressing the growth cone motility when active. However, as growth cone dynamics is strongly dependent on its Ca^{2+} concentration (Cohan et al., 1987; Henley and Poo, 2004), it is important to answer the question whether MS channels directly contribute to Ca^{2+} influx. Indeed, TRP channels have been shown to exist in the growth cones of cultured Xenopus neurons (Wang and Poo, 2005), rat cerebellar granule cells (Li et al., 2005) and rat hippocampal neurons (Greka et al., 2003). Whole-cell recordings from growth cones of Xenopus neurons showed that netrin-1 and brain-derived neurotrophic factor (BDNF) induced membrane depolarization and intracellular Ca^{2+} elevation even when all major voltage-dependent channels were blocked (Wang and Poo, 2005). This Ca^{2+} rise resulted in an attractive growth cone turning toward both netrin-1 and BDNF. Pharmacological blockade of TRP currents or down-regulation of Xenopus TRPC-1 expression abolishes both the netrin-1-induced Ca^{2+} elevation and the growth-cone turning. Thus, TRPC1-mediated currents contribute to the growth cone Ca^{2+} signaling that mediates the growth cone motility. As TRPC1 channel is mechanosensitive (Maroto et al., 2005), one can suggest that its modulation by membrane tension might be important for growth cone navigation.

The latter suggestion has been recently confirmed (Jacques-Fricke et al., 2006). Blockade of MS channels with the highly specific peptide GsMTx4 (Suchyna et al., 2000) strongly accelerated the rate of neurite extension both in cultures and within the intact spinal cord of Xenopus embryos. Interestingly, blockade of mechanosensitive TRPC1 channels with SKF-96365 produced the opposite effect, i.e. decreased the rate of neurite extension. Thus, several Ca^{2+} permeable MS channels appear to contribute to Ca^{2+} signaling in the growth cones and, moreover, the physiological response to Ca^{2+} elevation seems to be dependent on the source of Ca^{2+}. It is also of interest that only BAPTA, a fast Ca^{2+} chelator, but not EGTA, a slow Ca^{2+} buffer, inhibited growth acceleration induced by GsMTx4. As BAPTA chelates Ca^{2+} fast enough to reduce even local Ca^{2+} elevations, this data favors the suggestion that local rather than global Ca^{2+} signals determine the growth cone motility. Consequently, spatial heterogeneity of MS channel expression and/or membrane tension appears to be crucially important for neuronal development.

1.3.3 Neuronal Excitability

Potassium channels control neuronal excitability through influence over the duration, frequency and amplitude of action potentials. Potassium channels that are active at rest inhibit depolarization toward firing threshold, and thus suppress excitation. K2P can influence neuronal excitability by regulating background leakage of potassium ions and resting membrane potential. A large majority of the K2P channels behave as background K^+ channels, whereas the TREK/TRAAK K2P channels which are expressed in the CNS (Hervieu et al., 2001; Talley et al., 2001) require either a physical (stretch) or chemical stimulation to open (for review see (Honore, 2007; Maingret et al., 1999)). Original electrophysiological study (whole-cell and single channel recordings) performed on cultured neuronal cells prepared from either the mesencephalic or hypothalamic areas of fetal rat brain demonstrated that neurons express three types of mechanosensitive K^+ channels (Kim et al., 1995). Interestingly, all three types were inactive at rest. Mechanical stress or application of arachidonic acid caused a drastic increase in their activity. It was suggested that these K^+ channels may play an important neuroprotective role. Indeed, poly-unsaturated fatty acids completely inhibited neuronal cell death in in vivo models of cerebral global ischemia and temporal lobe epilepsy (Lauritzen et al., 2000). Electrophysiological experiments confirmed that the administration of poly-unsaturated fatty acids activated K^+ channels. As poly-unsaturated fatty acids reduced both frequency and amplitude of excitatory postsynaptic potentials, the activated K^+ channels appeared to be located both pre- and postsynaptically. In addition, expression of both TREK-1 and TRAAK channels has been confirmed immunohistochemically. Thus, mechanosensitive K2P channels mediate the neuroprotective action of poly-unsaturated fatty acids.

Interestingly, TREK-1 K^+ channels can not only suppress neuronal excitability, but also potentiate it. Recently, Bockenhauer et al., (2001) reported that TREK-1 phosphorylation by protein kinase A (PKA) can dramatically change TREK-1 response to voltage. When TREK-1 is not phosphorylated, it operates as an open rectifier stabilizing the cell at resting potential. When TREK-1 is phosphorylated, it becomes a voltage-dependent K^+ channel, which only passes significant currents above a threshold. As changes in its open probability are half maximal above 0 mV, TREK-1 in this state will not influence initial depolarization of the cell to the action potential threshold, but will facilitate the recovery and repetitive firing. Thus, TREK-1 can also increase neuronal excitability.

Excitability of most neuronal cells is determined not only by their intrinsic membrane properties, but also by synaptic inputs they receive. Many neurons receive continuous, or "tonic", synaptic inputs, which increases their membrane conductance, and modifies the spatial and temporal integration of synaptic inputs (Hausser and Clark, 1997; Semyanov et al., 2004; Cavelier et al., 2005). In cerebellar granule cells, the spillover of synaptically released GABA gives rise to a persistent conductance mediated by GABAA receptors that modifies the excitability of granule cells (Brickley et al., 1996). This tonic conductance is mediated by α6 and δ-subunits containing GABAA receptors. Surprisingly, genetic ablation of α6 and δ-subunits of GABAA receptors eliminated this tonic conductance, but it did not affect the response of granule cells to excitatory synaptic inputs. It was shown that an increase in a "leak" voltage-independent K^+ conductance mediated by two-pore domain K^+ channels compensated the loss of tonic inhibition mediated by GABAA receptors (Brickley et al., 2001). Similar results were observed in the rat inferior collicular neurons after bilateral deafening. The decreased activity from deafness resulted in a significant decrease in the number of neurons expressing various two-pore domain K^+ channels, including stretch-activated TREK-2 channels (Cui et al., 2007). The deafness-associated decrease in TREK-2 expression was transient and significant only at 3 days, but not at 3 weeks and 3 months, following deafness. It was suggested that decrease in cell size (Sie and Rubel, 1992; Lustig et al., 1994; Niparko and Finger, 1997) and/or flattening of synapses (Gulley et al., 1978; Ryugo et al., 1997) can influence TREK-2 activity and expression. These results show that the physiological reliability of neuronal networks is fine-tuned by both extrinsic and intrinsic mechanisms underlying neuronal excitability functioning in concert.

1.3.4 Synaptic Function

Synaptic release is regulated by the variety of channels (for short review see (Rahamimoff et al., 1999)). Originally, stretch-induced increase of spontaneous transmitter release has been demonstrated at neuromuscular synapses (Fatt and Katz, 1952; Hutter and Trautwein, 1956). Increase in both frequency of miniature end-plate potentials and evoked end-plate potentials were observed in

response to muscle stretching (Chen and Grinnell, 1997). It was shown that 1% muscle stretch results in a 10% increase in release. Although the kinetics of this effect are very rapid favoring an involvement of stretch-activated channels, the stretch-induced enhancement of end-plate potentials was not dependent on extracellular Ca^{2+} and was not blocked by Gd^{3+}, a non specific blocker of MS channels (Chen and Grinnell, 1995; Grinnell et al., 2003). Unfortunately, most of central synapses are too small to be approached with a patch pipette. Therefore, there is no data on the possible role of MS channels in neurotransmitter release in the CNS. However, axonal expression of TREK-1 has been immunohistochemically observed in axonal fibers in stria medullaris (Hervieu et al., 2001). TRAAK is expressed in axons of cerebellar granular neurons (Lauritzen et al., 2000) and in neurons projecting their axons to the hippocampus via dorsal hippocampal commissure (Reyes et al., 2000). Moreover, TRP channel-mediated Ca^{2+} influx, presumably via mechanosensitive TRPC1 channels, has been shown in isolated nerve ending from murine neocortex (Nichols et al., 2007).

In contrast to a presynaptic role of MS channels, their possible postsynaptic contribution to GABAergic synaptic transmission in the cerebellum has been recently reported (Chavas et al., 2004). In cerebellar inter-neurons, both application of GABAergic agonists and repetitive stimulation of GABAergic afferent fibers induced somato-dendritic rise of intracellular Ca^{2+} concentration. This Ca^{2+} rise was not dependent on the activity of voltage-gated Ca^{2+} channels and/or release of Ca^{2+} from intracellular stores. In addition, muscimol application increased the dimensions of dendrites, and muscimol failed to induce a Ca^{2+} elevation in hypotonic solution. These results suggest that tension associated with GABA/GABAergic agonist-induced volume increase is the main driving force for the consequent Ca^{2+} increase. Unfortunately, direct involvement of stretch-activated channels in GABA-induced Ca^{2+} response has not been demonstrated, albeit a possible contribution of TRPV4 channels has been proposed. However, the observed link between GABAA receptor activation and local Ca^{2+} elevation allows suggesting a new pathway of inter-cellular signaling. Ca^{2+} response elicited by local hypo-osmotic stress and/or GABA release, leads to the release of amino acids (Kimelberg et al., 1990b; Franco et al., 2000) which will activate GABAA receptors in neighboring cells (either neurons or glial cells), where it will again trigger an osmotic stress and further amino acid release. In this way a wave of calcium and neurotransmitter release can propagate over an extended brain area.

1.4 Neuro-Glial Functional Network

The dominant role of neurons in information processing in the brain is challenged. In the recent concept of brain functioning, glial cells play active rather than passive, structural role. This working model regards the brain as dynamic

network of internally connected glial syncytium and synaptically connected neuronal circuits (Volterra and Meldolesi, 2005; Bloom, 2000; Sykova and Chvatal, 2000). In the frame of this concept, intercellular communications are not exclusively attributed to synaptic transmission executed through chemical and electric synapses, but can be also mediated by volume transmission. The latter is a slow and global pathway for cellular communications, which takes either extracellular (diffusion of messengers in the extracellular space) or intracellular (diffusion of signaling molecules in the cellular syncytium connected by gap junctions) route. As Ca^{2+} ions mediate functioning of both synaptically-connected neuronal networks (by controlling release of neurotransmitters and neurohormones) and glial syncytium (long-range signaling by propagating Ca^{2+} waves), Ca^{2+} signaling system seems to be of crucial importance for integration of glial and neuronal networks. MS channels are expressed on both neurons and astroglial cells and can therefore be an important element to integrate both networks.

Neuronal activity in the nervous system leads to accumulation of K^+ ions in the extracellular space (Lux and Neher, 1973). Once released, K^+ diffuses not only via the extracellular space but also through the glial syncytium, a process termed spatial K^+ buffering (Orkand et al., 1966). In the hippocampus, TREK-2 channels have been shown to be involved in both neuronal K^+ release and glial K^+ buffering induced by electrical stimulations or local K^+ ionophoresis (Pasler et al., 2007).

Sharp local increase in the extracellular K^+ concentration can initiate spreading depression. The spreading depression is a wave of severe neuronal depolarization that spreads through the grey matter. This phenomenon is exclusive for the CNS and appears to involve both the neuronal and the glial cells. The precise mechanism of the spreading depression is not fully understood, and its initiation presumably results from several factors acting in concert (for review see (Smith et al., 2006; Martins-Ferreira et al., 2000)). Interestingly, both electrical and mechanical stimulation can evoke the spreading depression (Leao, 1944). Therefore, neuronal stretch-activated K^+ channels are potentially capable to initiate extracellular K^+ elevation after mechanical stimulation. On the other hand, K^+ uptake into glial cells results in cellular swelling and may activate MS channels in glial cells and, in turn, K^+ release from glial cells into the extracellular space. Therefore, astrocytes may initially buffer K^+ and dampen the spreading depression but after reaching a ceiling, for example swelling-induced activation of K2P channels, they can amplify it and contribute to its propagation.

TREK-1 channels have been shown to be involved in the pathophysiology of depression. In mice, deletion of TREK-1 gene led to animals resistant to depression in five different modes (Heurteaux et al., 2006). As TREK-1 ablation affected serotoninergic transmission, serotonin-mediated regulation of TREK-1 functioning has been suggested to alter mood. However, the impact of neuronal or astrocytic TREK-1 remains to be investigated.

1.5 Conclusions and Perspective

The contribution of mechanical factors to the formation of the brain was recognized in classical studies. Investigating principles of brain morphology, His (His, 1874) explained the cerebral shape by unequal growth, competing volume demands, and resulting tension of different brain structures. Due to a tremendous success of genetic approaches, physical aspects of brain development remained almost not investigated during the last decades. However, recent morphological and modeling data suggest that mechanical forces may have a substantial impact on the corticogenesis (Hilgetag and Barbas, 2005). This study has shown that: (1) the characteristic shape of cortical convolutions can be explained by the minimization of axonal tension in cortico-cortical projections; (2) mechanical forces resulting from cortical folding strongly influence the relative and absolute thickness of cortical layers in gyri and sulci; (3) folding forces may affect the cellular migration during development. In addition, extremely fast, stretch-activated growth of integrated axons, i.e. axons without growth cones, has been demonstrated recently (Pfister et al., 2004). This and other collected data shows that mechanical forces can be of pivotal importance for morphogenesis and wiring in the CNS (Trepat et al., 2007; Van Essen, 1997).

The proposed tension-based hypothesis of the brain development suggests that neuronal and glial processes are under tension. As CNS tissue lacks a rigid structural framework to prevent the tension-induced shortening of cells, tension must be counterbalanced by hydrostatic pressure. Therefore, transport mechanisms that regulate osmotic balance, including MS channels, can contribute to this dynamic force equilibrium. Consequently, to understand the impact of endogenous tension on brain morphology and functioning, it is important to determine both the mechanical properties of CNS tissue (see for example (Lu et al., 2006)) and spatial distribution of mechanosensitive receptors/channels. Given the growing evidence that MS channels are different in terms of their mode of activation, it becomes even more important to identify the exact physiological stimulus that activates the channel in specific situations. Discovery of selective modulators of mechanically-gated channels in combination with new techniques allowing monitoring/modifications of membrane tension of normally operating cells will definitely bring more understanding to the field.

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Chapter 2
Ion Channels with Mechanosensitivity in the Nervous System

Gehoon Chung and Seog Bae Oh

Abstract Although sensation of the physical touch is the most ancient sense of all, our understanding is very scarce. After brief history of mechanosensation and the criteria of mechanical transduction channels are introduced, various ion channels with mechanosensitive properties are followed. Selective TRP channels, two pore domain potassium channels with mechanosensitivity and amiloride sensitive sodium channels are discussed in terms of their expression, biophysical properties, functional significance and their limits. Other possible mechanisms of mechanosensitivity with or without involvement of ion channels are briefly mentioned. Various methodologies frequently adopted to study the mechanosensitivity of the ion channels are classified by direct and indirect mechanical stimulation.

Keywords Mechanical Transduction · TRP · TREK · K2P · stretch · ion channels

2.1 Introduction

2.1.1 Tissues Whose Fundamentals are Mechanosensitivity

Life is going on within the physical world, so we cannot talk about biology without considering its physical context. Every organism, from single cell amoeba to human, makes every effort to understand the physical environment it confronts. Of the five senses of Aristotle, we have gathered a good amount of information on how we perceive light in the eye, a less but considerable information on how we hear sound by ears and we have a big outline of how we smell and taste. However, we still do not have a clue on the detailed molecular mechanism how we sense touch in the skin. Moreover, if we go into the details, considering the sensation of tympanic membrane movement is the fundamental

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of hearing, we do not yet know the very nature of hearing. Mechanosensation is probably the most ancient sensation required to maintain the life on earth. Yet, it is amazing how vague our knowledge on touch sensation is.

Sensing of physical touch in the skin is not all of what the mechanosensation does in our body. Just as mentioned, hearing is another form of sensation of mechanical force, and keeping the balance of the body requires mechanosensation as well. Detecting osmolar change is another form of mechanosensation. Sensation of the blood pressure by the aortic arch is very crucial in maintaining the cardiac muscle to contract within a certain range, and the autoregulation of vessel tone requires the sensation of blood flow. The muscle spindle is an example of the organ that cannot be talked without mechanosensation. Bone loses its mass if a certain amount of physical load is not applied, and there is a hypothesis that mechanosensation of the odontoblasts in the teeth causes the hypersensitive teeth (Brannstrom et al., 1967). In this chapter, we will discuss the history of study and the various ion channels found to have mechanosensitivity followed by the introduction of various methodologies used in the study.

2.1.2 History of Study

Study of sensitivity to the mechanical stretch of neuronal membrane was first started when Katz observed the electrical transduction of membrane stretch in the muscle spindle of frog (Katz, 1950). Next major step toward the nature of stretch activation of ion channel was 28 years later when Brown et al., (1978) reported the ion selectivity and reversal potential of the crayfish stretch receptor. Single ion channel conductance of stretch-activated cation channel (SAC) was first reported by Guharay and Sachs (1984) in embryonic chick skeletal muscle. In 1995, Awayda et al., (1995) reported the stretch activation of renal epithelial Na^+ channel protein expressed in planar lipid bilayer, which was the first demonstration of that the stretch activated channel could be activated without the help from any other cellular machinery. Identifying the nature of the stretch activated channels has been on the center of the study; however, it has not been very successful because of the lack of information on their molecular structure and their activation mechanisms, although there are several candidate molecules.

The first mammalian stretch-activated neuronal channel cloned was background K^+ channels with four transmembrane segments (Maingret et al., 1999a). The murine cDNA of TREK-1, a K^+ channels with four transmembrane domains and two pore domains, was cloned and transfected on COS cell to successfully reproduce stretch activated current. However, it is questioned what the physiologic meaning the K^+ channel would have, since the activation of the K^+ channel would lead to the hyperpolarization of the membrane potential.

Then there came the transient receptor potential channels, the large family of cation channels with versatile mode of activation. TRP channels gained the attention when a genetic screening for the mechanosensation genes in *Drosophila* revealed that the mutation of five 'nomp' genes made the bristles of the fly non-sensitive to the mechanical stimulation (Kernan et al., 1994). One of them, nompC, was found to express a TRP channel homologue protein, which was later named as TRPN1 (Walker et al., 2000). The major drawback of TRPN1 as a mechanosensitive channel is that it is not expressed in all species, and it is entirely absent in mammals. So the quest became searching for another channel with a properties similar to TRPN1 but with ubiquitous presence in mammals. Genetic screening of TRP channels with a conductance about 100 pS and high permeability of Ca^{2+} led to the discovery of TRPA1 in mouse utricle hair cells (Corey et al., 2004). TRPA1 even shared the ankyrin repeats of the TRPN1, which had been proposed as a possible molecular spring needed for the gating of the channel by stretch. Every circumstance favored TRPA1 as the sole mechanotransduction channel of the hair cell until two independent groups developed TRPA1 knockout mice and observed normal hearing capability (Bautista et al., 2006; Kwan et al., 2006).

Another big candidate of the mechanosensitive channel came from the genetic screening of the *C. elegans* for the genes involved in osmotic sensation. OSM-9 of the *C. elegans*, which was related to TRPV1 in mammals, were found important in the avoidance behavior of the osmotic stress, and searching for its mammalian counterpart led to another TRP channel, TRPV4. TRPV4 is expressed in most tissues where regulation of osmolarity is important for their functions or one of their important functions, such as brain, kidney, and endolymphatic duct of the ear. The channel is activated by osmotic cell swelling and by fluid flow, as well as by heat and phorbol esters and shows a high Ca^{2+} permeability and a large conductance of 90 pS. Moreover, the TRPV4 knockout mice have a deficit in hearing (Tabuchi et al., 2005). However, since the transgenic mice slowly developed hearing impairment after the age of 8 weeks, rather than born deaf, it is suggested that TRPV4 might not be the essential protein of hearing, but an accompanying one. TRPV4 might function in balancing the ionic gradient of the inner ear rather than being a direct mechanotransduction channel, and its deletion would result in the gradual disturbance of ionic balance and accompanying gradual loss of hearing (Tabuchi, et al., 2005).

2.1.3 Criteria for the Mechanosensitive Channels

The criteria for a channel to be believed as the mechanically gated one is well reviewed by Christensen and Corey (2007). First of all, to be considered as a candidate responsible of mechanotransduction, the candidate molecule must be expressed in the tissues where mechanotransduction is the main function, and the expression of the molecule during the developmental stage must be matched

to the time at which the mechanotransduction function in the tissues appears. Moreover, the blocking of the protein expression by knockdown or knockout technique should eliminate the mechanotransduction function of the tissue. The second is that the channel should be activated by mechanical force even when expressed in a heterologous expression system or liposome. If the protein is capable of mechanotransduction without other accessory proteins, this should not be the problem. The third criterion is that the candidate protein must be either a pore-forming subunit or a force-sensing subunit.

Three criteria that distinguish the direct activation by mechanical force from indirect activation were proposed in the same review. First, if a direct mechanical force gates a channel, the latency should be less than 5 milliseconds. This should not be the problem if a channel is activated by a direct stimulus, without the second messenger system. However, some of the stimulating methods frequently used, such as stimulation by osmolarity, are not suitable for the observation of the fast response. Thus, it is hard to distinguish whether the activation of TRPV4 by hypotonic solution is by a direct effect or by the second messenger system. The second criterion is that the kinetics of channel activation should depend on the amplitude of the stimulus, meaning that the larger mechanical force would elicit a faster channel opening. The third criterion is that there should be an observable physical movement, which could be correlated to the channel opening. Movement of the ciliary tips of the hair cells by sound is a good example.

2.2 Ion Channels with Mechanosensitivity

Ion channels shown to exhibit sensitivity to mechanical stimulation include transient receptor potential non-selective cation channel superfamily, two pore domain potassium channel family, amiloride sensitive sodium channel family and voltage-gated ion channels.

2.2.1 TRP Family

Transient receptor potential or TRP channels are a family of diverse ion channels with non-selective permeability to cations. Most TRP channels are comprised of six membrane-spanning helices with intracellular N- and C-termini. TRP channels are named so because they were first discovered in mutant *Drosophila* that, instead of having prolonged depolarizing after potential, has only a transient photoreceptor activity in response to prolonged intense light (Minke, 1977). TRP channels have been extensively studied as cellular sensors of environmental changes because they are activated and regulated by a wide variety of physical and chemical stimuli, including temperature, membrane potential, cell volume, osmolarity, membrane stretch, and ligands. They are divided, in mammals, into

six families: TRPC (Canonical), TRPM (Melastatin), TRPV (Vanilloid), TRPA (Ankyrin), TRPP (Polycystin) and TRPML (Mucolipin), based on amino-acid homologies. TRPC, TRPM and TRPV subfamilies consist of seven, eight and six different ion channels, respectively, whereas TRPA subfamily has only one mammalian member. TRPP and TRPML families are not fully characterized (Alexander et al., 2007; Clapham, 2003).

The first evidence for an involvement of TRP channels in mechanosensation came from mutations of the *Caenorhabditis elegans*, in which osm-9 gene encoding TRPV-like channels was deleted. The worm with mutated channels exhibited defects in the avoidance reaction to high osmolarity and nose touch (Colbert et al., 1997). TRPA family is of particular interest because it possesses tandem repeats of ankyrin domain in its N-terminus, which is believed to behave as a molecular spring required to be gated by stretch (Lee et al., 2006; Sotomayor et al., 2005).

2.2.1.1 TRPC

Members of transient receptor potential canonical (TRPC) family fall into four subfamilies: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5, by sequence homology and functions. All members of TRPC family were initially proposed as store-operated channels (SOCs), which meant they were activated by depletion of intracellular calcium stores. However, there were some conflicting evidences supporting that those were receptor-operated channels and insensitive to the store depletion. TRPC1 and TRPC6 are engaged to have mechanical sensitivity.

TRPC1

Transient receptor potential canonical 1 (TRPC1) is a mammalian homologue of *Drosophila* TRP channel and one of the earliest cloned human TRP channels (Wes et al., 1995; Zhu et al., 1995; Zitt et al., 1996). It is reported to be widely expressed in animal cells including brain, heart, testis, ovaries, smooth muscle, endothelium, salivary glands and liver, mostly by studies using RT-PCR (Beech, 2005). There were some reports of its expression in protein level in cerebellum and cortex (Goel et al., 2002), and nodose ganglion neurons (Glazebrook et al., 2005). In its early studies, it was considered to be a store operated channel, which means the channel opens when the intracellular Ca^{2+} in the endoplasmic reticulum is depleted. Later, however, it was reported that the channel was also activated by various stimuli such as the activations of metabotropic glutamate receptor mGluR1 and orexin OX_1 receptors, membrane stretch, 1-oleoyl-2-acetyl-sn-glycerol (OAG) and phospholipase C gamma.

Activation of TRPC1 by membrane stretch was first studied in *Xenopus* oocytes, in which their typical mechanosensitive cation channels reconstructed on liposome were stained by antibody of TRPC1, and their typical activity was increased with heterologous expression of human TRPC1 expression and

abolished with injection of TRPC1-specific siRNA (Maroto et al., 2005). However, stretch activation of TRPC1 was disputed in recent paper, in which pressure-induced cation influx was observed in vascular smooth muscle cells from TRPC1$^{-/-}$ knockout mice (Dietrich et al., 2007). There is possibility of other stretch activated channel taking over the role of the deleted TRPC1, and more definite evidences are requested to rule out the stretch activation of TRPC1.

TRPC6

Human TRPC6 has been found in various tissues including lung, placenta, ovary, spleen and other smooth muscle enriched tissues, such as stomach, colon, esophagus and myometrium (Beech et al., 2004). While TRPC6 has not been found in the nervous system in human, murine TRPC6 was found in brain (Boulay et al., 1997; Hofmann et al., 2000). There were conflict opinions of whether TRPC6 is store-operated or receptor-operated, however, a study with arterial smooth muscle, where vascular tone has to be tightly regulated with the pressure from the blood flow, provided an evidence that the channel might be pressure activated. Treating arterial smooth muscle with antisense oligodeoxynucleotides to TRPC6 decreased the expression of TRPC6 protein, and reduced the muscle depolarization and constriction in response to pressure (Welsh et al., 2002).

Recently a paper described the stretch-activation of TRPC6 by application of hypotonic bath solution and showed that the newly developed stretch activation blocker, tarantula peptide, GsMTx-4, inhibited the receptor activation of TRPC6 by diacylglycerol as well, suggesting that the stretch activation of TRPC6 might undergo the same pathway as receptor activation. (Spassova et al., 2006).

2.2.1.2 TRPV

TRPV subunits contain three to five ankyrin repeats in their N-termini and can be activated through a variety of mechanisms. TRPV1 to TRPV4 are well known as molecular heat sensors.

TRPV1

TRPV1 channels are found in the central nervous system and the peripheral nervous system and are involved in the transmission and modulation of pain, as well as the integration of diverse painful stimuli. TRPV1 is activated by a wide variety of physical and chemical stimuli including temperature greater than 43°C, low pH, anadamide, H-arachidonoyl-dopamine, and capsaicin. There are several reports suggesting a mechanosensitive role of TRPV1 in the kidney and bowel. However, mechanosensitivity of neuronal TRPV1 has not been studied so far.

The first evidence of the mechanosensitivity of TRPV1 was altered bladder voiding behavior of *trpv1*$^{-/-}$ knock-out mice (Birder et al., 2002). The bladder and urothelial epithelial cells from the genetically engineered mice showed markedly diminished secretion of ATP upon stretch, compared to their wild type counterparts. TRPV1$^{+/+}$ bladder cells secret ATP as a signal transmission molecule to neurons when mechanically stretched. The idea was supported by that the increased bladder activity due to activation of TRPV1 by anandamide in rats was blocked by capsazepine or resiniferatoxin, two potent TRPV1 antagonists (Dinis et al., 2004). This experiment was mimicked in patients with neurogenic detrusor overactivity (NDO) who showed marked increase in TRPV1 immunoreactivity. Resiniferatoxin decreased the firing rate of suburothelial TRPV1 nerve fiber in the NDO patients (Apostolidis et al., 2005).

The irritable bowel syndrome is a common functional disorder with chronic abdominal pain, discomfort, and abdominal bowel function, without any abnormalities seen on routine clinical testing. TRPV1 has been proposed to be related to the mechanical hyperalgesia associated with the abdominal discomfort experienced by patients. Single unit analysis of afferent fiber of jejunum from TRPV1$^{-/-}$ mice showed downward shift of the pressure-response curve compared to the wild type controls, and wild type controls showed decreased spontaneous firing rate when treated with capsazepine (Rong et al., 2004).

Recently, Naeini et al., (2006) reported that *trpv1*$^{-/-}$ mice secreted profoundly less antidiuretic hormone (ADH) in systemic hypertonic situation. The magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus from the knock-out animal, which are responsible in secreting vasopressin, did not show responses to hypertonic stimulation.

Despite the relative shortage of direct evidence, studying the roles of TRPV1 as mechanically activated channel protein is promising because of following two reasons. First, in *C. elegans*, OCR proteins, homologs of TRPV1, are necessary for mechanosensation. OCR1-4 proteins combine with OSM-9, a homolog of TRPV4, to make functional mechanosensitive channels. Multimer of OSM-9 and OCR-2 mediate mechanosensitivity in ASH neuron, and multimer of OSM-9 and OCR-4 forms mechanosensitivity of OLQ neuron of *C. elegans* (Kaplan and Horvitz, 1993). Second, mechanical allodynia and hyperalgesia with lowered mechanical threshold are associated with up-regulation of TRPV1 in the bladder and the intestines (Birder, 2007; Holzer, 2004). Immunoreactivity of TRPV1 was increased in the trigeminal ganglion when an experimental neuropathy induced mechanical hyperalgesia in the orofacial area of the rats (Kim et al., 2008). Up-regulated TRPV1 might be just a sensitizer of afferent neurons thereby lowering the mechanical threshold, but it is possible that TRPV1 is the mechanical transduction channel, thus increased expression of TRPV1 makes neurons more prone to the mechanical stimuli.

TRPV2

TRPV2 was initially reported in sensory neurons of rats and known as a temperature-gated channel with threshold of 52°C (Caterina et al., 1999). Mechanosensitive nature of TRPV2 in neuronal system has not been studied so far. However, TRPV2 expressed in vascular smooth muscle was reported to be activated by membrane stretch. In search for the molecular mechanism underlying blood flow regulation of vascular smooth muscle, Muraki et al., demonstrated the expression of TRPV2 in vascular smooth muscle cells, and observed the nonselective cation channel current and the elevation of intracellular calcium concentration by stimulation with hypotonic bath solution. Chinese hamster ovary cells transfected with TRPV2 showed similar results as the vascular muscle cells and treatment of mouse aorta with TRPV2 antisense oligonucleotides exhibited reduced nonselective cation channel current and intracellular calcium concentration. (Muraki et al., 2003)

TRPV4

TRPV4 was cloned from mouse kidney in search for the mammalian homolog of OSM-9, a TRP-like channel protein responsible for the detection of environmental osmolar change in *C. elegans* (Strotmann et al., 2000). Ever since TRPV4 was cloned, a number of studies were attempted to elucidate its relation to the osmolar change or the cellular swelling due to osmolar change.

TRPV4 is a polymodal receptor channel activated by moderate heat, arachidonic acid metabolites, phorbol esters and hypotonicity (Cohen, 2005). It is still on debate how these various stimuli activate TRPV4 and how these stimuli are related to each other. Whereas a decrease in osmolarity as little as 30 mOsm can activates TRPV4, direct membrane stretch does not influence TRPV4 (Strotmann, et al., 2000). Intracellular and extracellular calcium concentration was reported to modulate the gating of TRPV4 (Strotmann et al., 2003; Watanabe et al., 2003). However, the activation of TRPV4 by phorbol ester derivative 4α-Phorbol 12,13-didecanoate (4α-PDD), which does not activate protein kinase C, suggested the possible endogenous lipid ligand (Watanabe et al., 2002). A study with mutation on tyrosine residue near the N terminus of TM3 revealed that there are two distinct pathways of activation (Vriens et al., 2004). The Y555A/S556A double mutant TRPV4 expressing HEK293 cells showed decreased basal intracellular calcium concentration and did not responded to 4α-PDD or temperature over 42°C but was still activated by arachidonic acid, 5'6'-epoxyeicosatrienoic acid or hypotonic stimulation.

Two opposite results were published regarding mechanism underlying activation by hypotonicity. Xu et al., observed that hypotonicity resulted in tyrosine phosphorylation of TRPV4 on Tyr-253 residue by SRC family kinase, both in heterologously expressed TRPV4 in HEK293 and in murine distal convoluted tubule cell line. This phosphorylation was shown only when the channel was activated by hypotonic stress, and the overexpression of one of the

Src family kinases, Lyn, resulted in enhanced phosphorylation of TRPV4 both in basal and hypotonic stimulated status. Several Src family kinases were assessed to be physically linked to TRPV4 by means of biochemical and immunolocalization experiments (Xu et al., 2003).

Another group, Nilius et al., debated the role of phosphorylation of Tyr-253. They used Tyr-253 mutant TRPV4 to transfect HEK293 cells and still observed hypotonicity-induced calcium influx. They instead focused on the role of phospholipase A_2 (PLA_2) in activation of TRPV4 by hypotonic stress. Inhibitors of PLA_2 blocked TRPV4 activation in response to hypotonic stress but not in response to the 4α-PDD (Vriens, et al., 2004). These data complied with earlier reports that hypotonicity releases arachidonic acid by activating phospholipase A_2 (Basavappa et al., 1998; Pedersen et al., 2000; Thoroed et al., 1997). The same group also reported that arachidonic acid indirectly activated TRPV4 by formation of 5',6'-EET by P450 epoxygenase in a membrane-delimited manner (Watanabe, et al., 2003). By applying inhibitors of P450, Nilius et al., observed reduced elevation of intracellular calcium concentration by cell swelling. Taken together, reduced swelling induced activation of TRPV4 by inhibition of P450 or PLA_2 are consistent with the idea that hypotonic environment activated PLA_2 and the product arachidonic acid subsequently activates P450 to produce 5',6'-EET and this finally opens the TRPV4 channels. TRPV4 activation by heat and 4α-PDD was not affected by application of inhibitors of P450.

The role of TRPV4 as an osmotransduction channel was confirmed by behavioral tests with tail flinch counting. Intradermal injection of hypotonic solution increased the number of tail flinching behavior of the rats, as much as nine fold when the animal was sensitized by injection of PGE_2 and this effect was diminished by pretreatment of rats with TRPV4 antisense RNA (Alessandri-Haber et al., 2003). Intradermal injection of 2% hypertonic NaCl solution yielded similar results; the number of flinch was increased by injection, even more with sensitization by PGE_2, and TRPV4 antisense RNA diminished the increase by half. 10% hypertonic NaCl, however, showed different pattern in which TRPV4 antisense RNA was not effective in reducing the enhancement, suggesting that TRPV4 might not mediate nociceptive behavior induced by severe hypertonicity. The results were reproduced with transgenic mice, in which the increased time of licking and shaking of the paws by injection of 10% NaCl solution was not significantly different between $TRPV4^{-/-}$ mice and wild type group. Only the hypotonic solution and the mild hypertonic 2% NaCl solution induced different nociceptive behavior between two groups (Alessandri-Haber et al., 2005).

There are several reports about interactions of TRPV4 with other proteins. While hypotonic stimuli activated TRPV4 and resulted in elevation of intracellular calcium concentration in normal human airway epithelial cells, activation of TRPV4 by hypotonic stress was defective in those of cystic fibrosis patients. Activation of TRPV4 by 4α-PDD was still effective, and rescued the defective RVD of the patients in airway epithelial cells from CF patients (Arniges et al.,

2004). This result suggested that cystic fibrosis transmembrane regulator (CFTR) protein was necessary for TRPV4 to respond to hypotonic stress.

Another important protein involved in swelling of a cell is aquaporin, and the relationship between aquaporin and TRPV4 was studied in salivary gland (Liu et al., 2006). TRPV4 and aquaporin 5 were detected at the same location in the mouse submandibular gland acinar cells, and deletion of either of two genes resulted in reduced calcium entry and loss of regulatory volume decrease (RVD) by the hypotonic stimulation. Expression of N terminus-deleted AQP5 suppressed the activation of TRPV4 and subsequent RVD but swelling of the cells remained intact. This result suggests that interaction of TRPV4 with N terminus of AQP5 is crucial in activation of TRPV4 by hypotonic stimuli.

Third example of protein-protein interaction is with the dynamin-mediated endocytosis regulating protein, PACSIN3 (Cuajungco et al., 2006). No member of PACSIN protein family interacted biochemically with TRPV1 and TRPV2, and co-expression of PACSIN3, but not PACSINs 1 and 2, was functionally effective in membrane trafficking of TRPV4. Mutational study revealed that the interaction required proline rich domain upstream of the ankyrin repeats of the TRPV4 and the carboxyl-terminal Src homology 3 domain of PACSIN3.

Last protein-protein interaction was observed in primary cilium of kidney with TRPP2, which belongs to TRP polycystin family (Giamarchi et al., 2006).

Despite all these conflicting and incomplete data, role of TRPV4 as an osmotic or mechanical transducer is promising because defective avoidance behavior of hypertonic stimuli and nose touch of osm-9 deleted *C. elegans* was rescued by transgenic expression of TRPV4 (Liedtke et al., 2003). A mutational study confirmed TRPV4 as a functioning ion channel that the point mutation in the pore-loop of TRPV4, M680K, greatly reduced the rescue. TRPV4 failed to rescue the defective sensation introduced by other TRP channels of *C. elegans*, such as OCR-2.

2.2.1.3 TRPM

Unlike TRPC and TRPV sub-families, TRPM subunits do not contain any ankyrin repeat motifs in their N-termini, and entire functional motifs are in their C-termini. TRPM3, TRPM4, and TRPM7 has been related to mechanosensitivity.

TRPM3

TRPM3, third member of TRP melastatin family, exist in many splice variant forms and was found mainly in kidney, and within the brain, cerebellum, choroid plexus, locus coeruleus, the posterior hypothalamus, and the substantia nigra (Harteneck and Reiter, 2007). A long splice variant form with 1555 amino

acids was heterologously expressed on HEK293 and resulted in spontaneous influx of Ca^{2+} and this effect was enhanced with application of hypotonic solution and diminished with hypertonic solution (Grimm et al., 2003; Lee et al., 2003; Oberwinkler et al., 2005).

TRPM4

Earley et al., (2004), the same group that provided the evidence of TRPC6 being pressure transducer of vascular smooth muscle, found TRPM4 mRNA in vascular smooth muscle from cerebral arteries and reported that attenuating the expression of TRPM4 with antisense oligonucleotides also reduced pressure-induced depolarization. However, this provide only circumstantial evidence and the direct evidence and the exact mechanism remained to be elucidated.

TRPM7

TRPM7, which is an ion channel with serine/threonine-specific protein kinase motif in its C-terminus, was reported to translocate to cell membrane in response to sheer stress generated by laminar flow (Oancea et al., 2006). Treated with small interfering RNA (siRNA) targeted to TRPM7, human epithelial HeLa cells, which endogenously expresses TRPM7, showed decreased rate of cell volume recovery after osmotic swelling (Numata et al., 2007b). This result was again confirmed by TRPM7 transfected HEK293 cells (Numata et al., 2007a).

2.2.1.4 TRPA

Ankyrin repeats are an amino-acid motif believed to function in protein recognition. They contain antiparallel α-helices that can stack to form a superhelical spiral. Extrapolated ankyrin stacks composed of more than 24 repeats formed a full superhelical turn and was shown by atomic force microscope to have spring properties (Lee, et al., 2006). One of the mammalian transient receptor potential channel family, TRP ankyrin or TRPA, has 17 ankyrin repeats within its N-terminus (Sotomayor, et al., 2005). Its only member, TRPA1 has been shown to be the possible molecular sensor responsible of mechanical transduction of inner ear hair cells, but later was disputed by behavioral study with mutated animals.

TRPA1

Inner ear hair cells are the very essential structure of auditory, vestibular, and lateral-line sensory systems and it has long been speculated by those with otolaryngological interest and by those looking for mechanical transducer ion channels. Direct mechanical deflection of the hair bundles resulted in graded

responses up to 15 mV in amplitude, and occasional action potentials were also observed (Hudspeth and Corey, 1977). The extraordinary short period of latency suggested the direct gating of ion channel by sound, as comparable to the long latency period of photoreceptor with second messenger system (Corey and Hudspeth, 1979). After 20 years of stacking up the evidences, such as high Ca^{2+} permeability, conductance of around 100 pS, and permeability to the fluorescent lipophilic dye FM1-43, the full story explaining the mechanism under hearing sensation was awaiting one last piece of the puzzle to come, the actual ion channel directly gated by mechanical stimuli. Then TRPA1 was found expressed at the tips of the hair bundles of receptor cells in the inner ear, by in situ hybridization and immunohistochemistry (Corey, et al., 2004). Functional data supported the idea with fulfilling all the description previously made. The level of TRPA1 mRNA in the mouse utricle increases markedly at E17, coincident with the appearance of the utricular hair cell transduction, and treatment with small interfering RNAs targeted to TRPA1 resulted in decreased transduction current. The authors even suggested that the fast adaptation property of TRPA1 could be the mechanism underlying cochlear amplifier, a process termed for the vibration of the cochleae's basilar membrane. TRPA1 became the strongest candidate of mechanical transducer ion channel, until the same group reported a contradictory results with TRPA1 knockout mice (Kwan, et al., 2006). TRPA1 was known as a polymodal ion channel activated by noxious cold below 17°C and pungent compounds derived from mustard oil, cinnamon oil and garlic. While TRPA1 deleted mice showed reduced avoidance to cold, chemical, and mechanical stimuli, they showed normal startle reflex to loud noise, normal sense of balance, a normal auditory brainstem response, and normal transduction currents in vestibular hair cells.

Another group published similar but not identical results using different strain of TRPA1 deleted mice (Bautista, et al., 2006). The transgenic mice did not respond to the application of mustard oil, garlic, bradykinin and acrolein, an ingredient of tear gas, suggesting TRPA1 was the sole mediating receptor of these chemicals. However, TRPA1-deficient mice displayed normal cold sensitivity and unimpaired auditory function, suggesting TRPA1 was not required to detect noxious cold or sound.

A possible explanation for these results with knock-out mice is that other mechanical transducing channels might be up-regulated and compensate for the deleted TRPA1.

2.2.1.5 TRPP

Polycystic kidney disease (PKD) proteins, which found mutated in autosomal dominant polycystic kidney disease (ADPKD), was recently renamed as TRP polycystin family because of its 6-transmembrane TRP-like channel structure. PKD1 has not been successful in producing convincing evidence of forming a channel by itself, and other members of TRPP family have not been studied enough so far, except for TRPP2, formally PKD2.

TRPP2

No connection of TRPP2 to the nervous system has been established, however, its exclusive expression in the cilia of renal epithelial cells makes it very promising as a functional sensory channel responding to shear-stress from fluid flow (Alenghat et al., 2004; McGrath et al., 2003; Nauli et al., 2003; Pazour et al., 2002).

2.2.2 K_{2P} Family

Potassium channels are one of the earliest identified ion channels and comprise the most diverse family in terms of both structure and function. The number of transmembrane domains within their structure classifies them as those with two transmembrane domains, four transmembrane domains and six transmembrane domains. Four transmembrane domain potassium channels were recently cloned and its structure was very different from those of the other previously known K^+ channel. While other K^+ channel families had only one pore domain, surrounded either by six transmembrane domains or by two transmembrane domains, this K^+ channel with four transmembrane domains consists two pore regions, hence it is also called K_{2P} (Lesage et al., 1996). K_{2P} became of special interest because they were shown activated by membrane stretch and cell swelling (Fink et al., 1998; Lesage et al., 2000; Maingret et al., 1999b; Patel et al., 1998). Currently, K_{2P} comprise fifteen members.

TWIK-1, which stands for tandem of P domains in a weak inward rectifier K^+ channel, was the first four transmembrane domain potassium channel cloned, and a related K^+ channel with 4 transmembrane domains, TWIK-1 related K^+ (TREK-1) channel, was cloned from a mouse brain library (Fink et al., 1996). Despite the similar overall structure to the TWIK-1 channel, TREK-1 channel generated an outwardly rectifying current, different from that of inwardly rectifying TWIK-1. The third and fourth members of two pore domain K^+ channels (K_{2P}) were subsequently identified and named after their function, which were TASK for TWIK-related acid-sensitive K^+ channel and TRAAK for TWIK-related arachidonic acid-stimulated K^+ channels (Duprat et al., 1997; Fink, et al., 1998). TREK-2, which shares 65% amino acid sequence identity with TREK-1, was added to the list after two years (Bang et al., 2000).

2.2.2.1 TREK1

TREK-1 is a polymodal K^+ channel and is by now the most extensively studied member of two pore domain K^+ channels. Human TREK-1 is highly expressed in the brain, with particular abundance in GABA-containing interneurons of the caudate nucleus and putamen. Other areas where TREK-1 was found are prefrontal cortex, hippocampus, hypothalamus, midbrain serotonergic neurons

of the dorsal raphé nucleus and sensory neurons of the dorsal root ganglia, and peripheral tissues such as the gastrointestinal tract and odontoblasts (Honore, 2007; Magloire et al., 2003). Each subunit of TREK-1 consists of four transmembrane with both the amino and the carboxy terminal domains facing the cytosol. Two subunits combine to form a functional channel, with possibility of heterodimerization. Variety of physical and chemical stimuli regulates TREK-1, which include membrane stretch, intracellular acidosis, warm temperature, and arachidonic acid. Recently, intracellular phospholipids such as PIP_2 were shown to enhance basal channel activity (Chemin et al., 2005). Quenching of PIP_2 by polyamines switched TREK-1 off to the closed state, while elevation in membrane inner leaflet PIP_2 concentration increased the basal activity of the channel.

Membrane stretch activated TREK-1 channel in both cell-attached and excised inside-out patch clamp configurations, and opening of TREK-1 was independent of intracellular calcium and ATP levels, suggesting a direct gating mechanism of the channel. Moreover, negative pressure was more effective in opening the channel in inside-out patch configuration, indicating a certain direction of deformation had more advantage in opening the channel (Maingret, et al., 1999b; Patel, et al., 1998). Pressure-effect plotting was sigmoidal with half-maximal activation of 50 mmHg (Honore et al., 2006). Disruption of actin cytoskeleton increased the number of active channel, suggesting that the membrane tension was transmitted to the channel by lipid bilayer and cytoskeleton was rather a tonic suppressor (Lauritzen et al., 2005; Maingret et al., 2000). Conversely, overexpression of TREK-1 channel altered the cytoskeletal network and induced the membrane protrusions (Lauritzen, et al., 2005). Activation of TREK-1 by membrane stretch showed a fast desensitization within 100 ms and this desensitization was independent of the cytoskeleton, indicating the channel itself was the sole factor of desensitization (Honore, et al., 2006).

The carboxy terminus of TREK-1 seems to play a critical role in gating of the channel by mechanical stimulation, since progressive deletion of the carboxy terminus reduced the sensitivity of the channel to the mechanical stimulation, shifting the pressure-effect curve towards the more negative pressure (Maingret, et al., 1999b). A glutamate residue, E306, is of particular interest. Cytosolic acidification protonates the glutamate residue and the protonation of E306 changes the pressure dependency of the channels, rendering the channel active at atmospheric pressure (Honore et al., 2002; Maingret, et al., 1999b). Taking two observations together, Chemin et al., (2005) proposed a general modulating model of TREK-1, in which the interaction of negative charged glutamate residue and PIP_2 played a central role. Quenching PIP_2 by polyamine prevents the interaction of the positively charged residues of carboxy terminus with inner leaflet phospholipids, hence the channel become inactive to any stimuli. With PIP_2 in the membrane inner leaflet, the carboxy terminus partially bonds to the membrane and the channel is capable of activation by various stimuli. When the negative charged glutamate residue is substituted for the neutral alanine, other

residues with positive charge make a tight bond to the PIP_2 in the inner leaflet, thus the channel becomes locked open and behaves as a leak K^+ channel. Exogenous phospholipids and cytosolic acidosis works in similar manner.

So far, we have discussed the activation mechanism of TREK-1. However, it is a K^+ channel, which means that the activation of the channel will result in the hyperpolarization of the membrane potential, rather than depolarization. For example, activation of TREK-1 by volatile general anesthesia agents was suggested to be their possible cellular mechanism, by the experiment in which halothane failed to anesthetize $Trek1^{-/-}$ mutant mice (Patel et al., 1999). Thus, it would be appropriate to mention the inactivation mechanism of the channel as well. Phosphorylation of the serine residue S333 locks the channel in a closed state (Patel, et al., 1998). Hence, in an inflammatory state, prostaglandin E_2 (PGE_2) and other inflammatory mediators inhibit TREK-1 channel by activating protein kinase A, and the cell becomes more sensitive to depolarization by opening of other transducing channels such as TRPV1 (Honore, 2007). Neurotransmitters acting on Gs- and Gq- coupled receptors are also capable of inhibiting TREK-1 by cAMP pathway. However, inhibition of TREK1 by membrane stretch has not been reported and it is still not clear what impacts the activation of TREK-1 by membrane stretch have within physiological range.

2.2.2.2 TREK2

While TREK1 mRNA is expressed in most tissues with particular abundance in the brain and the lung, TREK2 mRNA is expressed exclusively in the nervous system, including cerebellar granule neurons, dorsal root ganglion neurons and cortical astrocytes (Bang, et al., 2000; Gnatenco et al., 2002; Gu et al., 2002; Han et al., 2003, 2002; Kang and Kim, 2006). The TREK-2 channel shares all the functional properties of TREK-1 (Kim et al., 2001; Lesage, et al., 2000).

2.2.3 DEG/ENaC Family

Degenerin/Epithelial sodium channels (DEG/ENaC) are widely expressed from invertebrates to mammals. DEG/ENaC channels in vertebrate have been implicated in various functions such as nociception, sodium homeostasis, regulation of the composition and volume of lung fluids, mechanosensation, synaptic transmission, and memory and learning (Kellenberger and Schild, 2002). Involvement of DEG/ENaC to the mechanosensation is more prominent in *C. elegans*, whose mutant showed defect in mechanosensation. When two of the DEG/ENaC family found in the nematode, MEC-4 and MEC-10, are mutated, *C. elegans* becomes insensitive to touch (Chalfie and Au, 1989).

The story of DEG/ENaC began when Jorgensen and Ohmori (1988) reported that amiloride blocked the hair cell transduction channel of a chick.

When the α, β and γ subunits of the ENaC channel were cloned from rat colon, it gained immediate interest that they were part of a large gene family DEG whose members included MEC-4 and MEC-10 (Canessa et al., 1993, 1994). The MEC-4 and MEC-10 proteins belong to DEG family along with the related protein DEG-1, and are called degenerins because they cause degenerative neuronal death when certain residues are mutated. The DEG family in *C. elegans* and ENaC family were combined to yield a big family of ion channel and called DEG/ENaC channels.

Searching for the other ENaCs or related channels in mammals lead to the discovery of αENaC isoforms in the chick cochlea and another branch of the DEG/ENaC family in mammals, ASICs, acid sensing ion channels (Corey, 2006). A number of ASIC channel subunits were found in the mechanosensitive neurons of the dorsal root and trigeminal ganglia and sensory nerve endings in skin (Garcia-Anoveros et al., 2001).

Despite these intriguing findings, there are some drawbacks in the involvement of DEG/ENaCs in hair-cell mechanotransduction. The conductance of the DEG/ENaCs is too low to be regarded as the sole transduction channel in the hair cells (10–15 pS). The Na^+ selectivity is too high (P_{Na}/P_K = 5–100), and Ca^{2+} permeability is too low to fit within the biophysical properties of hair cell transduction channels ($P_{Ca}/P_K < 0.4$) (Kellenberger and Schild, 2002). Behavioral tests with knockout mice were not successful either. ASIC2 knockout mice had normal hearing, confirmed by auditory brainstem-evoked response (ABR) (Peng et al., 2004; Roza et al., 2004). ASIC1, ASIC3 and αENaC-deleted mice were not deficient in hearing, either (Rusch and Hummler, 1999; Xie et al., 2003).

2.2.4 Other Possibilities Involving the Mechanosensitivity

Gating of an ion channel involves conformational change of the protein structure. The mechanical deflection of lipid bilayer and the voltage difference around the cell membrane are alike in that they both can supply the energy needed for the conformational change. In that sense, modulation of the voltage-gated channels by mechanical force is not difficult to imagine. The mechanosensitivity of voltage-gated K^+ channel currents was successfully demonstrated in rat trigeminal ganglion neurons and in heterologous expression system. Altered activation and inactivation kinetics of *Shaker* K^+ channel was observed by stretching the membrane of oocytes transfected with *Shaker* K^+ channel (Gu et al., 2001; Laitko and Morris, 2004; Tabarean and Morris, 2002). However, Piao et al., (2006) demonstrated that hypotonic environment enhanced two types of the VGKC currents, both transient $I_{K,A}$ and sustained $I_{K,V}$, without affecting their activation and inactivation kinetics. Disrupting the actin-based cytoskeleton by cytochalasin D further increased the VGKC currents and the actin-based cytoskeleton stabilizer, phalloidin, decreased VGKC. Confocal images showed that

hypotonic stimulation also disrupt the actin-based cytoskeleton. The actin-based cytoskeleton was suggested to behave as a barrier to the mechanical force imposed.

There has been a paper suggesting the role of purinergic receptor in the light touch sensation of the skin. *Xenopus laevis* oocytes acquired mechanosensitive features when $P2Y_1$ cRNA from sensory neurons was transfected. Touch to the frog sensory nerve fibers generated more action potential when P2 receptor agonists were present and less action potential with antagonists. $P2X_3$ cRNA induced less mechanosensitivity to the oocytes than $P2Y_1$ cRNA and P2X specific agonists failed to enhance the frequency of touch-induced action potential in the sensory fiber. $P2X_3$ and $P2Y_1$ receptors are also different in distribution. Whereas $P2Y_1$ mRNA was concentrated in large-fiber dorsal root ganglion neurons, $P2X_3$ mRNA was localized to small-fiber sensory neurons. Therefore, $P2Y_1$ receptor was proposed to participate in the non-nociceptive light touch sensation in the skin (Nakamura and Strittmatter, 1996).

Loss of bone mass because of the reduced physical load suggests that the bone is an exquisite mechanosensitive organ. The primary cilia of the surface of the osteoblasts and osteocytes, along with its putative role in sensing fluid flow in the kidney, provided a promising evidence of mechanosensitivity of bone cells. Moreover, polycystin 2, which is responsible for the flow sensing function of renal epithelial cells, is expressed extensively in the ciliary tip of the bone cells. However, the flow-induced Ca^{2+} flux in bone cells was independent of primary cilia, and chelating the extracellular Ca^{2+} did not inhibit the rise of the intracellular calcium concentration, suggesting that more complex mechanism might be involved in the mechanosensation mechanism of the bone cells (Malone et al., 2007).

There was a report suggesting that the bending of the artificial phospholipid bilayer by shear stress dissociated the alpha subunit and the beta and gamma subunits of the G protein, thereby activating the G protein without the receptor activation (Gudi et al., 1998). If this is the case, the cell membrane could be a mechanoreceptor by itself without help from all the channels mentioned throughout this chapter. Moreover, it could provide the possible molecular mechanism underlying the modulation of the mechanosensitivity of TREK-1 by PIP_2.

2.3 Experimental Setups

2.3.1 Methods of Applying Mechanical Stimulation

All experimental techniques such as patch clamping, calcium imaging, gene deleting, gene silencing, protein overexpression, and pharmacological methods can be used in the experiments on the mechanosensitivity of the ion channels. One difference from the experiments of other field is that the methods of

applying mechanical force have to be established. The mechanical force applied to the cells can be divided to the direct methods and the indirect methods.

2.3.1.1 Direct Methods

Direct methods comprise several means of stretching the cell membrane by direct application of physical force. The simplest way is to deflect the membrane surface by a water jet from pico-injector. This method does not need any additional instruments other than the conventional patch clamp experiment setup. All it takes is that the pipette tip used for pressure application of a drug is simply filled up with the bath solution being used. Whereas the tip should not be so close to the cell surface when applying drugs because the pressure from puffing pipette may affect the cell membrane, the tip should be close enough to make deflection on membrane surface. However, determining the distance between the pipette tip to the cell membrane is the major problem in this method. This distance is the two most important factor along with the pressure, yet the distance, especially the z-axis distance, is very hard to keep under certain range because in the microscope, the cell and the pipette tip are always viewed from the top.

Another method frequently used is actually touching the cell membrane with heat polished blunt tip of glass pipettes (Drew et al., 2002). A very sensitive piezoelectric manipulator is required for this method. Although different by the size of the cell tested, a sensitivity of 1 μm is desirable. This method needs an expensive instrument but the membrane deflection can be under precise control.

Whereas above two methods evoke a local distension of the membrane by poking, inflation of cell membrane by regulation of the pressure inside the glass electrode of the patch clamp experiment can be used to dilate the overall membrane directly (Cho et al., 2002). This technique is well suitable for the excised patch clamp experiments, enabling the single channel recording with or without the intervention from the other cellular components. On the other side, it cannot be used in calcium imaging experiment.

Culture dishes made of elastic silicon can be used in calcium imaging experiment. After the cells being studied were seeded on a silicon culture dish, the bottom of the culture dish was stretched under calcium imaging system. The stretch of the bottom of the silicon culture dish is transferred to the cells seeded on it, and the change of the intracellular calcium concentration is inspected with calcium indicating fluorescent dye. Although this technique is not difficult to perform overall, precise control of stretching is difficult. It cannot be used with patch clamp experiment.

Ferromagnetic beads can be used to stimulate cells mechanically (Wang et al., 1993). After ferromagnetic microbeads are attached to the extracellular matrix receptors, for example integrin beta 1, of the cells being tested, cells are placed under magnetic field and twisted or stretched by regulating the magnetic force. The strongest feature of this technique is that the cells are able to be

manipulated with various patterns, mimicking diverse force applied to the cells. High sensitivity and large dynamic range from pico Newton to micro Newton are other advantages this technique has. However, it cannot be used with ion channels expressed in the liposomes and it is not possible to be used in patch clamp experiments either.

2.3.1.2 Indirect Methods

Indirect methods of mechanical stimulation include alteration of osmolarity of the bath solution. Hypotonic bath solution tends to swell cells and the tension from the stretched membrane is transferred to the ion channels. However, it should be discriminated what really activated the ion channels. Membrane stretch might play the central role, but osmolar change itself might as well influence the channel, and the increased volume of the cell might be the sole activator of the ion channel. Another factor that needs a careful consideration in experiments with hypotonic solution is that when the cells are in whole-cell configuration of patch clamp experiment, the volume of the cell increases infinitely until it finally ruptures. When an unpatched cell encounters hypotonic environment, it takes up water from the solution until the equilibrium of the osmolarity of intracellular and extracellular fluids are reached. However, when the cell is in whole-cell configuration, the pipette solution becomes a large reservoir of solute so that the cell has to take up a lot of water to lower the intracellular tonicity, too much of the water than it can bear. The cell often ruptures before it gets equilibrium (Pedersen and Nilius, 2007).

Let us assume that an unpatched cell with a closed boundary, a finite volume and an osmolarity of 100 mOsm is bathed in a 20% hypotonic solution of 80 mOsm. The cell will immediately takes up water from surround until its osmolarity gets 80 mOsm. The volume of the water needed in this process can be calculated by a simple math, 100 divided by 1.25 equals 80. The volume of the water needed to make the equilibrium of the osmolarity is just 25% of the original volume of the cell, which means a 25% increase in the volume of the cell is enough to compete with 20% hypotonic solution. When there is a large reservoir of solute continuously supplying the cells, however, the water can hardly equilibrate the concentration of solute to the hypotonic extracellular solution. Even when the water comes in and the volume of the cell increases, the concentration of solute does not decrease significantly. The discrepancy between the continuous swelling of the cell and relatively constant intracellular osmolarity must be considered when studying the mechanosensitive ion channels by patch clamp with hypotonic solution.

Measurement of the cell volume is important when cells are stretched by hypotonic extracellular solution. Large-angle light scattering method is helpful to be used with intracellular calcium measurement experiment. The fundamental of this technique is that large particles scatter light in the near-forward direction while smaller particles scatter light in all directions. Evaluating the

change of scattered light is all it takes to measure the volume change of a cell (Pedersen and Nilius, 2007).

Adjusting the flow rate of the bath solution is a good substitute for the shear stress from blood flow, and has been used in many studies involving mechanosensitive ion channels of the vascular endothelial cells. This technique is also often used to study the mechanism underlying bone formation and resorption in the presence and absence of physical load.

2.3.2 Other Considerations

In order to test the mechanosensitivity of a channel, cells isolated from tissues where mechanosensitivity is their primary function or one of their important functions should be used. Heterologous expression system could be adopted to reproduce the mechanical transduction function of the channel; however, in order to be convinced as a real mechanosensitive one, the ion channel must be shown activated by stretch without any help from other intracellular compartments. Expressing the ion channel in an artificial lipid bilayer or liposome is often required to eliminate the contamination from other cellular elements. The agents that disrupt cytoskeleton might as well be used for the same purpose.

Cells with primary cilia or cells from the tissues whose important function is sensation of mechanical or osmotic stress are usually selected to study mechanosensitive ion channel. Good examples of such are inner ear hair cells, cells lining blood vessels, organum vasculosum lamina terminalis of the brain, cartilage, bone and renal cells. Primary cilia are solitary, immotile, microtubule-based organelles that grow and project from the cell surface in many vertebrate tissues. They are believed to function as flow sensors in kidney tubule epithelial cells. The organum vasculosum of the lamina terminalis is one of the circumventricular organs of the brain that function as systemic osmoreceptors located outside the blood brain barrier. An intimate response to the minute changes in blood pressure is the essential function of the arterial smooth muscle and it is a good source of study on the stretch activated channels.

Pharmacological isolation of mechanosensitive channels is not easy because of the lack of specific blocker. Although gadolinium and lanthanum were frequently used to block stretch activated channels, they were general TRP channel blockers, not specific to the stretch receptors. Recently, a novel stretch sensitive ion channel selective blocker, GsMTx-4, was isolated from the venom of the tarantula *Grammostola spatulata* (Suchyna et al., 2000). It is an approximately 4 kD peptide with a hydrophobic face opposite a positively charged face, active in both the D and L forms. The peptide has been successful in study of mechanosensitive reactions of heart, smooth muscle, astrocytes, and skeletal muscle. It is suggested that the mechanism of its inhibition of stretch sensitive channel involves interaction with the lipid bilayer, especially with the anionic

lipids (Jung et al., 2006). It is also reported that GsMTx-4 inhibited not only the stretch activation but also the receptor activation of TRPC6, suggesting that the stretch activation and receptor activation of TRPC6 might share a common path (Spassova, et al., 2006).

Understanding the function of mechanosensitive channels by genetic manipulation is not easy because of the redundant and overlapping functions of several ion channels, which provides an evolutionary evidence for the importance of mechanosensitive channels. Most of the mutational study on mechanosensation has been done with relatively simple organism, *C. elegans*. It is a nematode with transparent body of 1 mm in length and has been a model organism of molecular and developmental biology. The worm shows several mechanosensory behaviors, including response to touch, osmotic avoidance, and male mating behaviors (Bounoutas and Chalfie, 2007). *C. elegans* has many advantages over higher organisms for the study of mechanosensation. The entire genome of the worm has been sequenced and all 302 neurons have been identified. The worm is transparent so that manipulation of the neurons is easy. Indeed, the first touch insensitive mutant organisms were of *C. elegans* (Bounoutas and Chalfie, 2007).

2.4 Conclusion and Perspective

So far, we have discussed various ion channels expressed in several tissues whose important function is transduction of mechanical stimulation. Most of the stretch activated ion channels were studied outside the nervous system, but the results were not irrelevant, since the same ion channels are also expressed in the nervous system. Like the cliché, if something quacks like a duck in the backyard, it will quack like a duck in the front yard, and it will probably be a duck.

TRPA1 and TRPV4 are the most promising candidates and there are many evidences supporting them. However, data from behavioral experiments did not correlate with the other evidences supporting TRPA1. The existence of the other mechanosensitive channels in the hair cell compensating for the absence of TRPA1 could be possible since the evolution favors multiple copies for a vital function. By contrast, behavioral evidences supported TRPV4. However, hypotonic stimulation is not suitable for the observation of the direct channel gating with a fast latency, thus the mode and mechanism of the activation by osmolar change is still under investigation. The possibility of transduction through the second messenger system or other chemicals has to be ruled out for TRPV4 to be believed as an osmolar transduction channel. The mechanosensory role of the other TRP channels in the nervous system is yet to be studied with supporting evidences from the non-neural system. TREK-1 and related K^+ channels were successfully shown mechanosensitivity. However, it is yet to be determined how the opening of K^+ channels contributes to the generation of the action potential. Evidences supporting the ENaC as the mechanotransduction channel

are weak and the study is merely successful. However, ENaC has not been ruled out completely and more intensive study is required, either to rule out or to confirm the mechanosensitivity of ENaC.

There are many obstacles before the true participants or unifying mechanisms are understood. Looking back on the study of voltage gated ion channels, one can imagine how long it will take until we can say with confidence what the mechanosensitive channels are and how they work.

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Chapter 3
Mechanosensitive Cation Currents and their Molecular Counterparts in Mammalian Sensory Neurons

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Abstract Although all animals employ mechanical sensations to apprehend their external and internal environments, the molecular transduction mechanisms involved in the detection of mechanical stimuli remain obscure. Mechanoreceptive somatosensory neurons are responsible for the transduction of mechanical stimuli into action potentials that propagate to the central nervous system. The ability of these sensory neurons to detect mechanical information relies on the presence of mechanosensitive channels that rapidly transform external mechanical forces into electrical signals. In the few past years, genetic approaches coupled to functional studies have provided insights into the basic mechanisms by which the senses of touch and pain are transduced in mammals. This review summarizes the methodological approaches and properties of mechanically gated ion channels in mammalian somatosensory neurons.

Keywords Pain · touch · mechanosensory transduction · stretch · osmotic shock · mechanosensitive channels · TRP channels · ASIC · dorsal root ganglion

3.1 Introduction

In mammals, sensations coming from the skin (i.e., exteroception), muscles and joints (i.e., proprioception) and viscera (i.e., interoception) are mediated by the somatosensory nervous system. Primary somatosensory neurons are able to transduce a variety of thermal and nociceptive stimuli into action potentials that propagate toward the central nervous system. Most of these sensory neurons are also responsive to some forms of mechanical stimuli, and as such function as mechanoreceptors. The mechanical stimulation of these mechanoreceptors generates a variety of sensations, such as touch, pressure, vibration, proprioception and pain.

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Sensory ganglia contain both low threshold mechanoreceptors (LTMs), which respond to light touch, vibration or internally generated movements, and high threshold mechanoreceptors (HTMs or mechanonociceptors) that sense noxious levels of pressure. Classically, numerous specialized or encapsulated endings (e.g. Golgi tendon organs and neuromuscular spindles in skeletal muscles, Meissner, Pacinian, Merkel and Ruffini corpuscles in the skin) are LTMs that are sensitive to innocuous touch, pressure and vibration, whereas a subset of Aδ- and C-polymodal nociceptors responds to intense mechanical stimuli, and therefore, subserves mechanical pain sensation (Olausson et al., 2002). Mechanonociceptors have a very different mechanical response pattern than LTMs; they are not especially sensitive to moving stimuli but are instead selectively responsive to static indentation.

Neural signals of the somatic mechanosensation begin with the excitation of the mechanoreceptive sensory endings. It is thought that mechanical forces applied to the sensory endings directly open mechanosensitive (MS) ion channels that depolarize the terminal. However, because of the small size and inaccessibility of sensory nerve endings, advances in understanding mechanical transduction mechanisms have proven difficult and have depended on the development of in vitro models of transduction (McCarter et al., 1999). Recent work in several laboratories determined that sensory neurons express various types of MS cation channels with distinct biophysical properties and subcellular pattern of expression (Drew et al., 2002, 2004; Hu et al., 2006; McCarter and Levine, 2006; Coste et al., 2007). This review highlights some recent developments regarding properties of these excitatory MS currents and their putative molecular counterparts in mammalian sensory neurons.

3.2 Methodological Approaches of Studying Mechanosensitive Ion Channels

Mechanical forces may activate MS channels via multiple mechanisms such as membrane bilayer mechanics, physical coupling to intracellular or extracellular tethers and sensitivity to second messengers generated by MS receptors/enzymes (Hamill and McBride, 1996). These models are not necessarily mutually exclusive and MS channels may be activated by different stimuli. Owing to these multiple mechanisms of activation, recorded channels may vary depending on the technique considered. Therefore, in this section, we focus on the strategies and methods that enable monitoring mechanotransduction currents.

Several types of mechanical stimuli have been used to investigate MS channels, including piezo-driven pressure, patch membrane stretch, shear stress, osmotic challenges and amphipathic compounds (Fig. 3.1). All these strategies are based on membrane deformation (Fig. 3.1), yet each has the potential to recruit different populations of MS channels. For example, strategies that entail

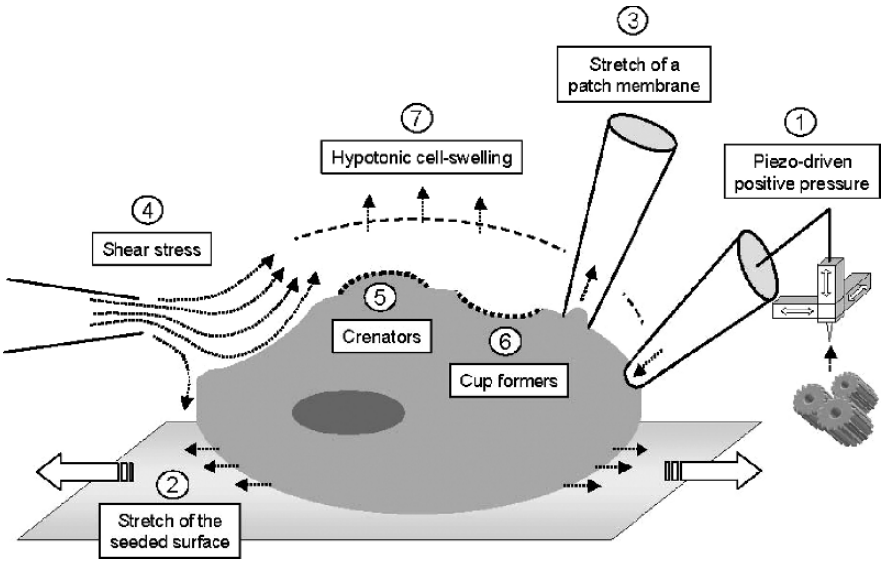

Fig. 3.1 Diagrammatic representation of strategies used to cause cellular mechanoresponsiveness A variety of mechanical stimuli can be applied to cells in vitro: (1) positive pressure using a piezo-electrically driven glass probe; (2) cell stretch caused by a flexible silicone elastomer; (3) stretch of the plasma membrane through a patch pipette; (4) fluid shear stress produced by changes in the flow or viscosity of the bathing solution; (5) cell-swelling caused by hypotonic conditions; (6) convex deformation of the membrane caused by anionic or neutral amphipathic compounds and (7) concave deformation of the membrane caused by cationic amphipathic compounds

membrane stretch via the application of positive or negative pressures to a patch electrode have revealed MS cation channels with persistent behavior (Cho et al., 2002). On the contrary, studies using piezo-driven mechanical probe have primarily revealed rapidly and slowly adapting cation currents (McCarter et al., 1999; Drew et al., 2002; Coste et al., 2007).

3.2.1 Piezo-Driven Pressure

Deformation of the plasma membrane of sensory neurons using a piezo-electrically driven fire-polished glass probe shows the most obvious analogy with mechanical somatodetection. The probe is typically positioned close to the cell surface at an angle $\geq 45^{\circ}$ in order to prevent transversal forces that could dislodge the cell from the substrate. The same software is generally used to drive the probe toward the cell and to record MS currents, allowing to control crucial parameters such as voltage and the intensity, velocity and duration of the mechanical stimulus. This technique, first developed in J.D. Levine's group,

has been applied to both cell bodies and neurites of sensory neurons in vitro (McCarter et al., 1999; Hu et al., 2006).

3.2.2 Cell Stretch

Some MS channels are activated by stretching the membrane bilayer. The opening of stretch-activated channels can be mediated either by fibrous proteins of the cytoskeleton or by physical changes in the lipid bilayer (Gottlieb et al., 2004; Suchyna et al., 2004). Two methods are commonly used to test for stretch sensitivity, that is, surface elongation of a flexible silicone elastomer substrate where cells have been seeded (Yuan et al., 2006) and application of positive or negative pressures to a patch membrane through a patch pipette (Hamill, 2006). The latter feature allowed the first recordings of stretch-activated ion channel currents more than 20 years ago (Guharay and Sachs, 1984).

3.2.3 Fluid Shear Stress

This technique consists in changing the physical properties of the superfusion solution. Two parameters can be modified to cause shear stress, (*i*) the perfusion flow and (*ii*) the viscosity of the solution.

Some cells are indeed sensitive to fluid-flow changes such as dorsal root ganglion neurons (McCarter et al., 1999; Takahashi and Gotoh, 2000) and some endothelial and epithelial ciliated cells, which respond to an apical fluid shear stress by an increase in intracellular Ca^{2+} (Praetorius and Spring, 2001; Giamarchi et al., 2006). Fluid shear stress can regulate the tissue morphogenesis, particularly the lumen diameter of kidney tubules and blood vessels (Davies et al., 1995; Tulis et al., 1998). The hypothesis that mechanical stimulation might be physiologically caused by changes in the mucus viscosity has been proposed for quite a while (Spungin and Silberberg, 1984). Recent studies confirmed that increasing the perfusion solution viscosity with 2–20% dextran, a complex branched polysaccharide, activates Ca^{2+} entry in ciliated epithelia (Andrade et al., 2005; Winters et al., 2007).

3.2.4 Osmotic Challenges

The plasma membrane of mammalian cells is more permeable to water than solutes. When the intracellular and extracellular media contain the same amount of osmolytes, they may be said isotonic with respect to each other. However, if one of them is less-concentrated, it may be said hypoosmotic and the net movement of water induced in the direction of the hyperosmotic compartment is called osmosis. The consequence of osmosis is a change in the

cell volume: hypotonic conditions induce cell-swelling, whilst hypertonicity causes cell-shrinkage. Thus, due to deformation of cell morphology and lipid bilayer tensions, osmotic variations are considered as a type of mechanical stimulation by many authors (Cunningham et al., 1995; Martinac, 2004; O'Neil and Heller, 2005; Lin and Corey, 2005).

Changes in osmotic pressure may affect most of mammalian cells and particularly sensory neurons that express a large panel of osmoregulated channels. Metabolic disturbances, cellular acidosis and hypoxia can increase the intracellular osmotic concentration (Nilius et al., 1996; Strange et al., 1996; Okada, 1997; Nilius et al., 1999). Marked changes in the extracellular tonicity are observed in dehydrated tissues, in edema, in abscesses (Wiese, 1994; Wiese et al., 1999) and in aquadynia (a cutaneous reaction to water) (Tsai and Maibach, 1999; Misery et al., 2003). In addition, injection of mildly hypertonic saline is widely used as an experimental model of muscle or joint pain (Alessandri-Haber et al., 2005).

A hypoosmotic challenge can be reproduced experimentally by adding water or a very low-concentrated solution to the extracellular medium (Boudreault and Grygorczyk, 2002; Kimura et al., 2004). Another possibility is to reduce (or increase in the case or hypertonicity) the external concentration of the main salt (generally NaCl) (Alessandri-Haber et al., 2005; Von Weikersthal et al., 1999). These methods have the disadvantage to alter the ionic strength and the electrochemical equilibrium. This can be avoided by keeping constant the ionic concentrations during the challenge and by adding (for hypertonicity) or omitting (for hypotonicity) the appropriated concentration of a neutral osmolyte such as D-mannitol or sucrose (Strotmann et al., 2000; Srinivas et al., 2003; Liu et al., 2006; Raoux et al., 2007).

3.2.5 Crenators and Cup Formers

There is still a debate on how mechanical stimulus is transferred to the MS channel: is the activating force coming directly from the lipid bilayer or is the force transmitted through cytoskeleton elements? In order to answer this question one can modify the bilayer properties. Indeed, pioneer work using amphipathic molecules on erythrocytes demonstrated that the two halves of the membrane bilayer can act as bilayer couples (Sheetz and Singer, 1974, 1976). Indeed, anionic and neutral amphipathic compounds such as free fatty acids, trinitrophenol and lysolecithin preferentially insert in the outer leaflet because of the natural asymmetric distribution of negatively charged phosphatidylserines in the inner leaflet. Consequently, these compounds induce the crenation of the erythrocyte plasma membrane. Conversely, positively charged amphipathic compounds such as chlorpromazine and tetracaine are expected to preferentially insert in the inner leaflet of the bilayer and cause the erythrocyte to form cup-shapes (Sheetz and Singer., 1974, 1976).

Such amphipathic molecules have been shown to activate the MS ion channel MscL from *Escherichia coli* with effectiveness proportional to their lipid solubility (Martinac et al., 1990). Moreover, the cationic or anionic amphipathic compounds were able to compensate for each other's effect. MS channels of the chick skeletal muscle are also directly activated either by such a convex or a concave deformation of the plasma membrane (Sokabe et al., 1993) and the activity of the recombinant mechano-gated two-pore-domain K^+ channels TREK1 and TRAAK is enhanced by crenators while it is inhibited by cup formers (Patel et al., 1998; Maingret et al., 1999). Thus, the bilayer couple hypothesis assumes that the stretch activation derives entirely from interactions within the bilayer and is independent of the cytoskeleton.

3.3 Biophysical Properties of Macroscopic Mechanosensitive Currents in Sensory Neurons

Detailed functional assay of MS currents is only feasible under conditions where the kinetics properties, ionic selectivity and mechanical sensitivity can be measured precisely, that are the mechanical load must be applied in a predictable way and the resulting MS currents should be measured with sufficient time and current resolution. To date, these conditions are only achieved by mechanically stimulating the cell body of sensory neurons while concurrently recording the mechano-gated current under voltage-clamp (McCarter et al., 1999).

MS currents evoked in sensory neurons have a relatively short latency, which argues against activation of a second messenger cascade and favors direct activation of a mechanically-activated channel (Hu et al., 2006). In response to sustained mechanical stimulation, MS currents decline or adapt, through closure of the transduction channels. Based on these adapting kinetics, 3 main classes of MS currents have been identified and loosely classified as either rapidly (RA), intermediately (IA) or slowly (SA) adapting (Fig. 3.2). A MS current that does not adapt within the testing range has been occasionally observed in sensory neurons (Drew et al., 2004; Coste et al., 2007). This non-adapting MS current exhibits the particularity to peak during the stationary part of the mechanical stimulus (Fig. 3.2). Interestingly, these different MS currents are differentially distributed in subsets of sensory neurons. The majority of large diameter sensory neurons, which are mainly LTMs, express RA MS currents, whereas small and medium diameter sensory neurons preferentially display SA/non-adapting MS currents (Fig. 3.2) (Drew et al., 2002, 2004; Hu et al., 2006; Coste et al., 2007). Differences in properties of MS currents among sensory neuron subpopulations *in vitro* are consistent with the in vivo physiological properties of LTMs and nociceptors (Lewin and Moshourab 2004).

Rapidly and slowly adapting MS currents in sensory neurons exhibit reversal potentials at about 0 mV and are carried by mechano-gated channels

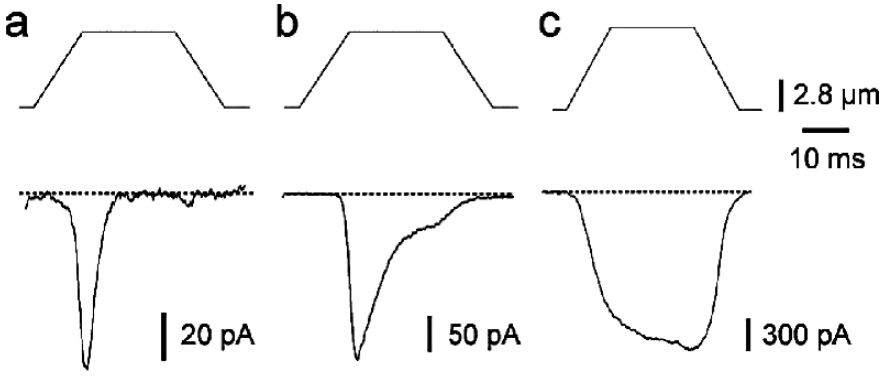

Fig. 3.2 Mechanosensitive currents evoked by mechanical ramp stimuli in sensory neurons Mechanosensitive currents showing rapid (**a**), intermediate (**b**) or no/slow (**c**) adaptations recorded in large, medium and small diameter sensory neurons, respectively. Mechanical ramp stimulations (top traces) were applied at a holding potential of –60 mV; the probe velocity was 850 µm.s^{-1}. (J. Hao, and P. Delmas, unpublished data)

nonselective for cations and impermeable to anions (Drew et al., 2004; McCarter and Levine, 2006; Coste et al., 2007). The similarity in the ionic selectivity of these MS currents among different populations of sensory neurons suggests that closely related ion channel subunits mediate these currents. The ability of RA MS currents to pass large organic molecules such as TEA, choline and Tris strongly favors the view that the pore of the underlying MS channels is relatively large (McCarter and Levine, 2006). At variance, however, Hu and Lewin (2006) suggested that the RA MS current reversed at very positive potentials ($\sim +$ 80 mV), suggestive of the participation of a pure Na^+ permeability (Hu et al., 2006).

While MS currents show the ability to be carried by Ca^{2+} and to a lesser extent by Mg^{2+}, both Ca^{2+} and Mg^{2+} at physiological concentrations produce a partial block of MS currents (Drew et al., 2002; McCarter and Levine, 2006). Although the mechanism of block is still unknown, it may be due to a Ca^{2+} binding site within the pore causing reduced permeation to Na^+.

Pharmacological studies of MS channels have been dominated by the use of unselective blockers such as metal cations. Not surprisingly, gadolinium (Gd^{3+}), a widely used blocker of various cationic conductances, blocked all MS currents in sensory neurons (Drew et al., 2002). Amiloride, another nonselective drug that blocks many epithelial Na^+ channels and TRPP-like channels (Kleyman and Cragoe, 1988; Delmas et al., 2004; Giamarchi et al., 2006), inhibited most MS currents at relatively high concentrations (Coste et al., 2007). These properties are shared by many mechano-gated cationic channels in other systems, including spider mechanoreceptor neurons, *Xenopus* oocytes and mammalian hair cells (Hamill and McBride, 1996; Höger et al., 1997). Recently, Drew et al., (2007) showed that a conopeptide analog termed NMB-1

(for noxious mechanosensation blocker 1) preferentially blocks SA MS currents in sensory neurons (Drew et al., 2007). Indeed, NMB-1 has an approximate 30-fold selectivity for MS channels mediating SA currents over those carrying RA currents. Biotynylated NMB-1 principally binds to peripherin-containing sensory neurons, which are typically considered as nociceptors. Consistently, animals show reduced mechanically evoked behavioral responses to high intensity mechanical stimuli in the presence of NMB-1. Collectively, these data reinforce the view that SA and RA MS currents are expressed in functionally distinct sensory neuron populations and are carried by different molecular entities.

3.4 Candidate Channels

Genetic screens for mechanosensory mutants in invertebrates such as *Drosophila* and *Caenorhabditis elegans* have identified several molecular candidates that are supposed to mediate MS currents in mammals. These proteins belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) and the TRP superfamilies.

3.4.1 The DEG/ENaC Superfamily

The first evidence of the involvement of DEG/ENaC channels in mechanotransduction came from works on the nematode *C. elegans*. Mutant animals defective in mechanosensitive behaviors allowed the identification of several genes of the DEG/ENaC superfamily that are essential for mechanical transduction. Two DEG/ENaC ion channel subunits called MEC-4 and MEC-10 are required for sensing light touch (Tavernarakis and Driscoll, 1997; Gillespie and Walker, 2001; Bounoutas and Chalfie, 2007). Together with MEC-6, these subunits form a mechanosensory complex including intra and extracellular tethers (Bounoutas and Chalfie, 2007).

3.4.1.1 Acid Sensing Ion Channels

Acid Sensing Ion Channels (ASICs) belong to an H^+-gated subgroup of the DEG/ENaC channel family of cation channels (Waldmann and Lazdunski, 1998; Lingueglia, 2007). Four ASIC genes have been cloned and encode seven channel subunits. Although all ASIC channel subunits are expressed in the peripheral nervous system, only ASIC2a, b and ASIC3 are specifically expressed in mechanosensory neurons. They are expressed in dorsal root ganglia and transported from the cell bodies to specialized mechanosensory structures such as Meissner and Merkel corpuscules as well as to penicillate and lanceolate nerve endings surrounding hair follicles. Both channel subunits are

also expressed in unmyelinated free nerve endings of the skin suggesting a role in the transduction of mechanonociceptive stimuli (García-Añoveros et al., 2001; Price et al., 2001).

In contrast to MEC-4 and MEC-10 in *C. elegans*, ASIC channel subunits express well in recombinant systems. However, recombinant ASIC channels failed to demonstrate any mechanosensitivity (García-Añoveros et al., 2001; Roza et al., 2004). Furthermore, the biophysical properties and pharmacology of these heterologously expressed ASIC channels differ from MS cation channels recorded in native sensory neurons (Lingueglia, 2007), suggesting that ASIC subunits may not contribute to the pore-forming subunit of the mechanosensory apparatus. In line with this, no differences in current amplitude or kinetics of MS currents were seen in ASIC2/ASIC3 null mutant mice (Drew et al., 2004).

The role of ASIC channels has been also investigated in behavioral studies using mice with targeted deletion of ASIC channel genes. ASIC2 knock-out mice exhibit a decreased sensitivity of both RA and SA LTMs (Price et al., 2000, 2001; but see Roza et al., 2004). By contrast, ASIC3 knock-out mice show increase in sensitivity of RA LTMs, no change in SA LTMs and reduced responses of myelinated HTMs to noxious stimuli. Although these data argue for the involvement of ASIC subunits in mechanotransduction, the definite demonstration that ASIC subunits carry MS currents in mammalian sensory neurons awaits further experiments.

3.4.1.2 Stomatin

In *C. elegans*, MEC-2 encodes an integral membrane protein with a stomatin homology domain involved in touch receptor function. MEC-2 exhibits a central sequence of 247 amino acids that possess 64% homology with the mammalian protein stomatin (Huang et al., 1995).

The use of mutant mice lacking stomatin like-protein 3 ($Slp3^{-/-}$) provided evidence to suggest that SLP3 is an important determinant of skin mechanoreceptor functions (Wetzel et al., 2007). About 36% of sensory neurons recorded *in vitro* show no responses to mechanical stimuli in $Slp3^{-/-}$ mice, compared with less than 5% in wild-type sensory neurons. Because the proportion of cells that normally display RA and SA MS currents decreased conjointly, one may hypothesized that SLP3 is necessary for both types of underlying MS channels. At the behavioral level, the loss of SPL3 impairs tactile discrimination capability and touch-evoked pain following neuropathic injury (Wetzel et al., 2007). Therefore SLP3 appears as an essential element of the mechanosensory apparatus in mammalian mechanoreceptors. Although its precise function is still unknown, SPL3 may be envisioned as a putative linker between MS channel subunits and the underlying microtubules, as proposed for its *C. elegans* homologue MEC-2 (Huang et al., 1995; Stewart, 1997).

3.4.2 The TRP Superfamily

Some of the most promising candidates for mechanically activated channels are members of the TRP superfamily, which is subdivided into 6 subfamilies in mammals (Nilius and Voets, 2005; Nilius et al., 2007) These channels play critical roles in sensory physiology, including requirements for thermosensation, olfaction, hearing and osmoregulation.

3.4.2.1 TRPV Channels

TRP channels of the vanilloid receptor (TRPV) group are best known for sensing heat and mediating neurogenic inflammation, but some have also been implicated in mechanosensation. TRPV1, which is activated by capsaicin, heat, acid and various lipids, is also required for normal stretch-evoked reflexes in the bladder (Birder et al., 2002) and for osmosensation in circumventricular neurons (Sharif Naeini et al., 2006). TRPV1 is strongly expressed in a subset of mammalian sensory neurons. However, it cannot directly sense and respond to mechanical stimuli (Gunthorpe et al. 2002) and *Trpv1*-null mutant mice exhibit normal acute mechanical sensitivity and mechanical hyperalgesic responses following tissue inflammation (Caterina et al., 2000; Davis et al., 2000).

Some evidence has implicated TRPV2 in aortic myocyte responses to membrane stretch and hypotonic stimulation (Muraki et al., 2003) and in constriction of blood vessels under increasing pressure (Davis and Hill, 1999). TRPV2 is preferentially expressed in large-diameter somatosensory neurons but its role in mechanotransduction remains to be tested (Caterina and Julius, 2001).

TRPV4 has been proposed to act as an osmosensor because, in addition to warm temperature and acidic pH, it is activated by cell-swelling through an indirect mechanism requiring fatty acid metabolites (Vriens et al., 2004; Liedtke, 2005). In that sense, TRPV4 cannot be considered as a real mechanosensor and there must be an upstream element to fulfill this function. TRPV4 is a crucial determinant in shaping the response of nociceptive neurons to osmotic stress (Alessandri-Haber et al., 2005; Liedtke et al., 2000). Disrupting *Trpv4* expression in mice has only modest effects on acute mechanosensory thresholds, but strongly reduces sensitivity to noxious mechanical stimuli (Liedtke and Friedman, 2003; Suzuki et al., 2003). In addition, TRPV4 has been implicated in mechanical hypersensitivity during inflammation (Alessandri-Haber et al., 2004, 2005).

3.4.2.2 TRPC1 Channels

Known as a store- and receptor-operated channel, the canonical TRP channel TRPC1 has been recently proposed to form the stretch-activated cation channel constitutively expressed in *Xenopus* oocytes (Parekh and Putney, 2005; Maroto et al., 2005). TRPC1 antisense reduces the endogenous stretch-activated

current in oocytes, whereas overexpression of TRPC1 increases the current (Maroto et al., 2005). Moreover, TRPC1 expression in CHO-K1 cells results in a 5-fold increase in MS cation current density (Maroto et al., 2005). Against the presumed role of TRPC1 in mechanotransduction, however, is the observation that this channel is widely expressed in mammalian cells, most being devoid of mechanosensory functions. Perhaps, the mechanosensitive signaling properties of TRPC1 rely on its interaction with other MS TRP subunits. Evidence has appeared to suggest that TRPC1 can interact with the putative MS TRPP2, a channel involved in mechanical detection of laminar fluid flow in renal epithelial cells (Tsiokas et al., 1999; Delmas et al., 2004; Delmas, 2004; Giamarchi and Delmas, 2006). Still to be tested is whether TRPC1/TRPP2 heteromers form functional channels that sense mechanical stimuli.

3.4.2.3 TRPA1 Channels

NompC, a member of the TRPN cation channel subfamily in *Drosophila*, together with its homologues in *C. elegans* and vertebrates, have been consistently implicated to play critical roles in mechanotransduction (Eberl et al., 2000; Walker et al., 2000; Sidi et al., 2003; Shin et al., 2005; Gopfert et al., 2006; Li et al., 2006). A unique feature of these TRPN-related channels is their large N-terminal domains harboring numerous ankyrin repeats. This prompted suggestion that these N-terminal domains may serve as tension transmission structures to the pore forming region.

TRPN channels are not present in the genome of reptiles, birds and mammals (Corey, 2006). The only mammalian TRP subunit with an extended domain of ankryin repeats is TRPA1. This subunit was suggested to form the main mechanotransducing channel of the inner ear (Corey et al., 2004; Nagata et al., 2005), but this proposal was not corroborated by knocking out strategies, since *Trpa1*$^{-/-}$ mice showed normal auditory responses and hair cell transduction currents (Bautista et al., 2006; Kwan et al., 2006). Recently, the *C. elegans* ortholog of mouse TRPA1 has been shown to be expressed in mechanosensory neurons and contributes to neural responses of these cells to touch (Kindt et al., 2007). In addition, mechanical pressure can activate *C. elegans* TRPA1 heterologously expressed in mammalian cells. These data demonstrate for the first time that *C. elegans* TRPA1 encodes an ion channel that can be activated in response to mechanical pressure.

The expression of TRPA1 in small diameter neurons of dorsal root and trigeminal ganglia in mammals led to the suggestion of its possible implication in mechanical pain sensation (Story et al., 2003; Nagata et al., 2005; Bautista et al., 2005). Consistent with its expression pattern in sensory neurons, mice lacking TRPA1 are deficient in the detection of acute high-threshold mechanical stimuli applied to the extremities (Kwan et al., 2006). However, contrary to these findings, the Julius group did not report deficit in responses to noxious mechanical stimuli in *Trpa1*$^{-/-}$ mice (Bautista et al., 2006). Kwan et al. (2006) went further on to demonstrate a role of TRPA1 in mechanical hyperalgesia, by

showing that mechanical pain threshold after bradykinin-induced inflammation is significantly higher in *Trpa1*$^{-/-}$ mice compared to wild-type (Kwan et al., 2006). Taken together, these data provide substantial evidence suggesting that TRPA1 mediates responses to high-threshold mechanical stimuli in nociceptive neurons. However, further investigations are clearly needed to determine whether TRPA1 is a real mechanotransducing channel and to clarify the existing discrepancies regarding its role in mammalian mechanosensation.

3.5 Conclusion

It is well known that mechanonociceptors and low threshold mechanoreceptors *in vivo* have different sensitivity and stimulus specificity. Although this may be explained in part by the geometry of the specialized terminal structures and interaction with support cells, this functional specificity provides strong hints that distinct transduction mechanisms underlay touch and pressure-evoked pain. However, unlike with other sensory transduction processes, there has been a lack of *in vitro* models to address these questions at the cellular and molecular levels. The development of new techniques that allow monitoring membrane tension changes while recording mechanotransduction currents was proved very useful in addressing many of these unresolved issues. It became clearly apparent that sensory neurons express MS currents with different mechanical threshold, adapting kinetics and pharmacology, raising the possibility that more than one mechanotransduction channel may be operating in subspecialized mechanoreceptive neurons.

What then are the candidate channels? Based on genetic studies in mammalian and non-mammalian species, members of the DEG/ENaC and TRP channel superfamilies are currently the more attractive candidates. Many fulfil some basic criteria for mechanosensitivity but most remain potential candidates. Even with candidate in hands, further studies will be required to fully elucidate their roles and the molecular mechanisms of mechanosensation.

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Chapter 4
Neurons and Cell Swelling-Induced Peptide Hormone Secretion

Vladimir Štrbák

Abstract Cell swelling (most often induced by extracellular hypotonicity or intracellular hypertonicity) evokes exocytosis of material stored in secretory vesicles resulting in a secretory burst of peptide hormones or enzymes from various types of cells. Swelling induces secretion from neuron axons, dendrites and bodies. Cell swelling-induced exocytosis possesses limited selectivity, cells specifically involved in water and salt regulation retain their specific response to osmotic stimuli; neurons of the hypothalamic supraoptic and paraventricular nuclei release oxytocin, vasopressin and angiotensin II and III in response to hyperosmotic stimulation. This atypical response related to their regulatory role could be obviated by $GdCl_3$ and at these conditions general unspecific response (exocytosis) to swelling-inducing stimuli emerged. It is concluded that swelling induced exocytosis is an ancient mechanism generally present in cells, in some of them is covered by specific response mediated by specific signalling. Swelling-induced hormone secretion could have pathophysiological implications especially when induced by ischemia.

Keywords Cell volume · swelling-induced peptide secretion · mechanosensitive receptors

Abbreviations **ANP**: atrial natriuretic peptide, **AVP**: andidiuretic hormone, vasopressin, **ME**: median eminence, **MNCs**: magnocellular neurosecretory cells, **NH**: neurohypophysis, posterior pituitary, **RVD**: regulatory volume decrease, **PVN**: hypothalamic paraventricular nucleus, **SIADH**: syndrome of inappropriate secretion of antidiuretic hormone, **SON**: hypothalamic supraoptic nucleus, **TRH**: thyrotropin releasing hormone, **TRP**: transient receptor potential channels.

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4.1 Introduction

Keeping volume within certain limits is a prerequisite for cell integrity, survival and proper function. Cell volume regulation in some cells is engaged regularly after changes in extracellular osmolarity experienced under physiological conditions (e.g. intestinal epithelial cells, kidney epithelial cells, brain cells etc). More commonly, cell volume perturbations occur as a consequence of changes of intracellular osmolyte concentration, and such changes are an integral part of many physiological processes: regulation of cell proliferation, apoptosis, cell migration, cell metabolism (for review see Hoffmann and Pedersen 2006) and secretion (Štrbák and Greer 2000). Metabolic activity leads to the generation of intracellular osmolytes, which tend to cause the cell to swell. Alterations in the extracellular tonicity can either promote cell shrinkage or swelling, depending on the direction of the osmolarity gradient. Most of the cell reactions to volume changes are aiming at the renewal of initial volume status. To drive water flux, which finally accomplishes cell volume adjustment, cells produce an osmotic difference between the intra- and extracellular spaces by inducing osmolyte transport. The defence against excess cell swelling is accomplished by a reduction of the intracellular osmolarity by release of organic- or inorganic osmolytes from the cell and by synthesis of osmotically less active macromolecules from their specific subunits (Jakab et al. 2002, Lang et al. 1998a,b, Wehner et al. 2003). To accommodate expanding volume during hyposmotic swelling, animal cells change their shape and increase surface area by drawing extra membrane from surface and intracellular reserves (Groulx et al. 2006). Exocytosis of intravesicular material should help a cell meet a relative extracellular hyposmotic challenge by expanding the plasmalemma through fusion with vesicular membrane (Štrbák and Greer 2000).

In higher organisms cell volume responses to osmotic challenge are integrated into a signal transduction network regulating various cell functions (Jakab et al. 2002, Lang et al. 1998a,b, Wehner et al. 2003) including apoptosis (Yurinskaya et al. 2005). Swelling-induced peptide secretion represents important cellular reaction when material stored in secretory vesicles is expulsed as a secretory burst of peptide hormones or enzymes from various types of cells including endocrine, neurons, leukocytes or those of the exocrine pancreas (for review see Štrbák and Greer 2000).

4.2 Cell Volume and Peptide Secretion

In general way cell swelling evokes and shrinking has most often suppressing effect on secretion of proteins and peptides. Dynamics (Fig. 4.1) of the secretory response to cell swelling closely resembles that induced by specific secretagogues (Strbak et al. 2004, Bacova et al. 2006a). Perifusion of pituitary cells with 10 nM TRH (prolactin natural secretagogue) as well as cell swelling induced by

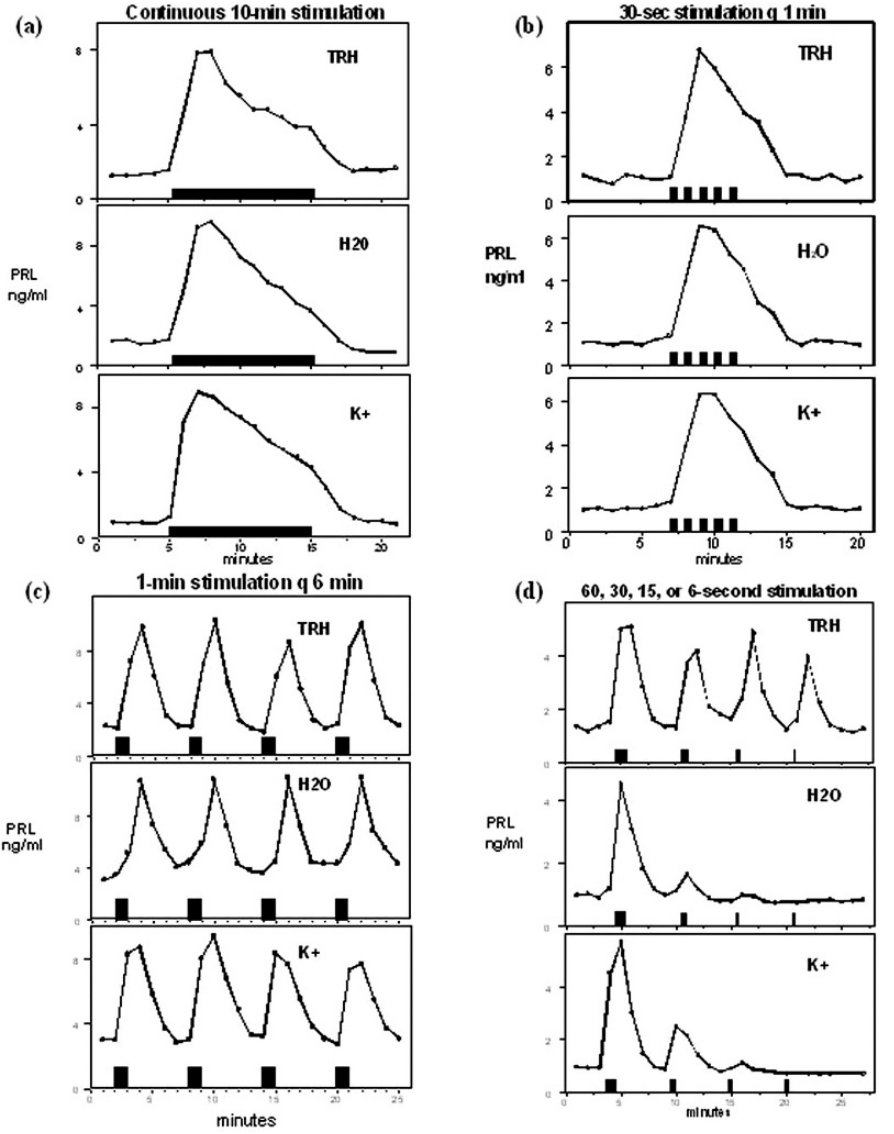

Fig. 4.1 Dynamics of prolactin secretory response of perifused pituitary cells to 10 mmol.L^{-1} thyrotropin releasing hormone (TRH, prolactin natural secretagogue), hypotonic solution (H_2O, medium diluted with 30% water) or 30 mmol.L^{-1} KCl (K^+, membrane depolarizing solution – unspecific stimulus). Continuous stimulation (**a**) or repeated stimuli lasting 30 sec with 30 sec interstimulus interval (**b**), 1 min stimulation with 6 min interstimulus interval (**c**) or stimuli lasting 6–60 sec with 6 min interval (**d**) were compared. Differences were found only if stimulus lasted less than 1 min. With permission from Strbak et al. (2004)

hypotonic solution (medium dilution with 30% H_2O) or depolarizing 30 mM KCl stimulates an immediate dose-related high-amplitude prolactin secretory burst, reaching a peak at 1–2 min followed by a decline to a low plateau within 5–10 min during continuous exposure to the same stimulus (Fig. 4.1a). Repeated stimuli with 30 sec. interstimulus interval produce the same secretory response as continuous stimulation (Fig. 4.1b). For all three types of stimuli, the secretory response to continuous exposure and refractory periods to repeated stimulation (less than 1 min) were essentially identical (1a, 1b and 1c). An identical high-amplitude secretory burst was induced by exposure to TRH for times varying from 6 to 600 sec. In contrast, for 30% H_2O and high KCl, the secretory amplitude was proportional to the exposure time between 6 and 60 sec (Fig. 4.1d). While the TRH response was triggered by rapid specific receptor binding, a very short pulse would not have time to produce sufficient transmembrane osmotic gradient or K^+ difference. It was concluded that hyposmotic medium does not trigger peptide release by the specific receptor-ligand binding (Strbak et al. 2004, Bacova et al. 2006a).

Although the mechanism of swelling-induced exocytosis is not clear, comparison of signal transduction mediating glucose- and hypotonicity-induced insulin secretion indicates the presence of a novel signalling pathway (Bacová et al. 2005). Hyposmotic stimulation of insulin secretion from pancreatic islets is independent from both the extracellular (Blackard et al. 1975, Straub et al. 2002, Bacová et al. 2005) and intracellular Ca^{2+} (Bacová et al. 2005), various insulin release inhibitors and ion-flux modifiers (Blackard et al. 1975, Straub et al. 2002), it cannot be inhibited by noradrenaline (Bacová et al. 2005), does not involve protein kinase C (Bacová et al. 2005), G proteins or phospholipase A_2 activation and is N-ethylmaleimide insensitive (Bacova et al. 2007). Interestingly, glucose itself induces swelling of pancreatic ß-cells (Miley et al. 1997) and tumour-derived INS-1E cells (Jakab et al. 2006); a consideration that swelling is integrated into glucose signalling is therefore fully justified.

Exocytosis of intravesicular material may help a cell to meet a relative extracellular hyposmotic challenge by expanding the plasmalemma (Štrbák and Greer 2000, Groulx et al. 2006). In contrast to electrolytes, export of proteins and peptides out of the cell does not change an osmotic balance between extra- and intra-cellular spaces insofar as to have a significant effect on the cell volume regulation. Regulatory volume decrease (RVD) dependent on the transport of osmolytes outside of the swollen cell and swelling-induced peptide secretion could be affected in an opposite way; when medium Ca^{2+} is depleted or Ca^{2+} influx prevented by Ca^{2+} channel blockers, the amplitude of cell swelling is greater and the RVD slower, in contrast, extracellular Ca^{2+} is not required and, in fact, negatively modulates osmotically-induced secretion in many cells (Greer et al. 1990, Štrbák and Greer 2000, Sato et al. 1991, Bacová et al. 2005). The apparent negative modulation by Ca^{2+} influx of osmotically induced secretion in normal pituitary cells (Greer et al. 1990) may simply be due to an augmented signal intensity resulting from the greater magnitude and

duration of cell swelling from an osmotic stimulus when Ca^{2+} influx is prevented than when it occurs.

The secretion induced by extracellular hyposmolarity is due to the relative reduction of extracellular osmolarity compared to the intracellular osmolarity and is not due to dilution of any essential extracellular elements. If culture medium is prepared by the addition of equivalent osmolar amount of mannitol (Greer et al. 1983), sorbitol (Blackard et al. 1975, Straub et al. 2002), choline chloride (Blackard et al. 1975) or by biologically inactive L-glucose (Nikodemova et al. 1999) to maintain isosmolarity on both sides of the plasma membrane, no secretion is induced. If the culture medium is made hyperosmolar, the peptide secretion is not stimulated and is often suppressed (Kucerova and Štrbák 2001, Miley et al. 1997, Najvirtova et al. 2003, Sato et al. 1990, Wang et al. 1992). However, on the return of cells to an isosmolar medium, there is a prompt dose-related burst of 'off response' secretion whose magnitude is proportional to that of the preceding hyperosmolarity (Greer et al. 1985). This indicates that it is the relative difference between intracellular and extracellular osmolar concentration which causes exocytosis rather than the absolute osmolarity of either compartment.

4.3 Cell Swelling Induced Peptide Secretion – Broad Unspecific Phenomenon

4.3.1 Secretory Response

The release of almost all protein and peptide hormones tested in vitro was stimulated in a concentration-related manner by medium hyposmolarity, or isosmolar medium containing permeant molecules from various tissues, including **pituitary**: gonadotropins LH and FSH, adrenocorticotropin, melanocyte-stimulating hormone, thyrotropin, prolactin, beta endorphin (Greer et al. 1990, Sato et al. 1991, Greer et al. 1985, 1983, Sato et al. 1990, Wang et al. 1989, Back et al. 2000), **pancreas**: insulin, (Blackard et al. 1975, Straub et al. 2002, Bacová et al., 2005), thyrotropin releasing hormone (TRH), (Benicky et al. 1997), and glucagon (Blackard et al. 1975), **hypothalamic and septal neurons**: gonadotropin-releasing hormone (Inukai et al. 1992), TRH (Najvirtova et al. 2003, Nikodemova et al.1999, Kucerova and Štrbák 2001, Najvirtova et al. 2002), **heart slices**: TRH (Bacova et al. 2006a), **cardiomyocytes**: atriopeptin (Greenwald et al. 1989) and **juxtaglomerular kidney cells** – renin (Skott 1986). Cell swelling induced exocytosis is not restricted to endocrine cells or hormones. Medium hyposmolarity also induces secretion of exocrine pancreatic enzymes (Blackard et al. 1975) and myeloperoxidase from human polymorphonuclear leukocytes (Sato et al. 1990).

The cell swelling as a nonspecific stimulator of protein/peptide release with limited selectivity (Bacová et al. 2005, Najvirtova et al. 2003) is a suitable tool

for characterization of peptide secretion from various cells and tissues (Štrbák and Greer 2000). We have used hyposmotic medium to stimulate TRH secretion from brain septum (Kucerova and Štrbák 2001), hypothalamic structures (Kiss et al. 2005) and heart slices, where its secretagogue has been unknown (Bacova et al. 2006a, Štrbák 2006).

4.3.2 Swelling Induces Secretion from Different Parts of the Neuron

As it was mentioned above, hypothalamic neurons respond to swelling by a neuropeptide release. It is of much interest to know – which parts of the neuron are able to secrete in response to swelling. To address this question the hypothalamic paraventricular nucleus, representing cell bodies in which thyrotropin-releasing hormone is synthesized, and the median eminence, representing nerve terminals, were incubated in vitro (Nikodemova et al. 1999). Various hypo- and hyperosmotic solutions were tested to determine osmotic sensitivity of thyrotropin-releasing hormone secretion. High KCl (56 mM) causing membrane depolarization was used as a non-specific control stimulus to induce thyrotropin-releasing hormone secretion. A 30% decrease of medium osmolarity (from 288 to 202 mOsmol/l) increased thyrotropin-releasing hormone secretion from both the paraventricular nucleus and median eminence. A 30% decrease of medium NaCl content by its replacement with choline chloride did not affect basal thyrotropin-releasing hormone secretion. Increasing medium osmolarity with biologically inactive L-glucose did not affect basal or KCl-induced thyrotropin-releasing hormone secretion from either structure. Medium made hyperosmotic (350–450 mOsmol/l) by increasing the NaCl concentration resulted in a concentration-dependent decrease of basal thyrotropin-releasing hormone secretion and abolished KCl-induced TRH secretion. If an osmotically equivalent amount of choline chloride was substituted for NaCl, there was no effect on thyrotropin-releasing hormone secretion, indicating a specific action of Na^+ (Nikodemova et al. 1999). This study indicates a secretory response to swelling and specific sensitivity to inhibitory action of high concentrations of Na^+ ions of both thyrotropin-releasing hormone-producing parvocellular paraventricular neurons and thyrotropin-releasing hormone-containing nerve terminals in the median eminence (Nikodemova et al. 1999). Similarly ethanol in isosmolar medium induces TRH release from the hypothalamic PVN and ME and posterior pituitary by a mechanism involving neuronal swelling (Najvirtova et al., 2002). It is concluded that swelling can induce secretion from different parts of neurons – similar release of the TRH was evoked from the hypothalamic paraventricular nucleus (PVN) (mostly perikarya) and the median eminence and posterior pituitary (exclusively axon terminals) (Nikodemova et al. 1995, 1999, Najvirtova et al. 2002).

4.4 Specific Response of Cells Engaged in Water and Salt Regulation to Osmotic Stimulation

Besides common unspecific cellular secretory responses to anisosmolarity (e.g. stimulation of peptide secretion by hypotonicity and inhibition by hypertonicity), it is also well established that direct stimulation of the hypothalamic PVN and supraoptic nucleus (SON) with hyperosmotic saline via microdialysis increases vasopressin (AVP, antidiuretic hormone) and oxytocin levels in extracellular fluid (Hattori et al. 1990, 1992, Morris 1990, Landgraf et al. 1990, Landgraf and Ludwig 1991) by mechanism involving axons, dendrites, and cell bodies (Pow and Morris 1989). Levels of these hormones increase also in plasma as a result of their release from axon terminals in the posterior pituitary. These neuropeptides are secreted within hypothalamic nuclei and into the blood in a Ca^{2+} -dependent manner (Hattori et al. 1990, Ludwig and Landgraf 1992, Soldo et al. 2004). The release of vasopressin from the neurohypophysis (NH) is regulated primarily by changes in extracellular fluid osmolarity; mechanism is synergistically controlled by osmoreceptors and neuropeptides released within the hypothalamus (Chakfe and Bourque 2000) – the response to excitatory peptides is blunted by reducing osmolality of extracellular fluid. Excitatory effects of angiotensin II, cholecystokinin and neurotensin facilitating secretion from magnocellular neurosecretory cells (MNCs) are mediated by stimulation of stretch-inactivated channels and are Gd^{3+} sensitive (Chakfe and Bourque 2000). It is of interest that also angiotensin II and III are released from PVN in response to local hyperosmotic stimulation (Qadri et al. 1994) suggesting that they play transmitter role in central osmoregulation. Immunoneutralisation by intracerebroventricular injection of antiserum against atrial natriuretic peptide (ANP) inhibited the increase of oxytocin secretion and content of oxytocin in the PVN, SON and NH induced by the osmotic stimulus (Chriguer et al. 2003). Thus, the increase in plasma oxytocin and in oxytocinergic neurons of the hypothalamus-posterior pituitary system in response to hypertonicity depends on the action of endogenous ANP, i.e., extracellular fluid hypertonicity must activate ANPergic neurons which directly or indirectly stimulate oxytocin release (Chriguer et al. 2003).

4.5 Inhibition of Specific Response

To shed more light on the mechanisms of specific and unspecific response, we have used tissue explants of hypothalamic PVN and SON and posterior pituitary to follow effect of simultaneous hyposmotic stimulation on thyrotropin releasing hormone (TRH) and oxytocin secretion (Najvirtova et al. 2003, Bacova et al. 2006b). Under our experimental conditions, both neuropeptides were simultaneously secreted into medium from the same tissue

explants, though most likely from different neurons: parvocellular and MNCs respectively.

As expected (Najvirtova et al. 2002, 2003; Kiss et al. 2004, 2005, Bacova et al. 2006b), TRH secretion from PVN and NH (Fig. 4.2, upper part) was stimulated by hypotonic medium while oxytocin, neurohormone engaged in water and salt regulation, was not released (Fig. 4.2 lower part, left parts of panels) after cell swelling-inducing stimulus (Najvirtova et al. 2003, Bacova et al. 2006b). This is in good agreement with observations that hypotonic stimuli hyperpolarize magnocellular neurosecretory cells via stretch inactivated channels (Bourque and Oliet 1997) and that ethanol inhibits the release of oxytocin from the posterior pituitary induced by high K^+ (Knott et al. 2000) or suckling (Subramanian 1999). Therefore, it was concluded that cell swelling-induced exocytosis possesses limited selectivity; cells specifically engaged in water and salt regulation retain their specific response to osmotic stimuli (Najvirtova et al. 2003).

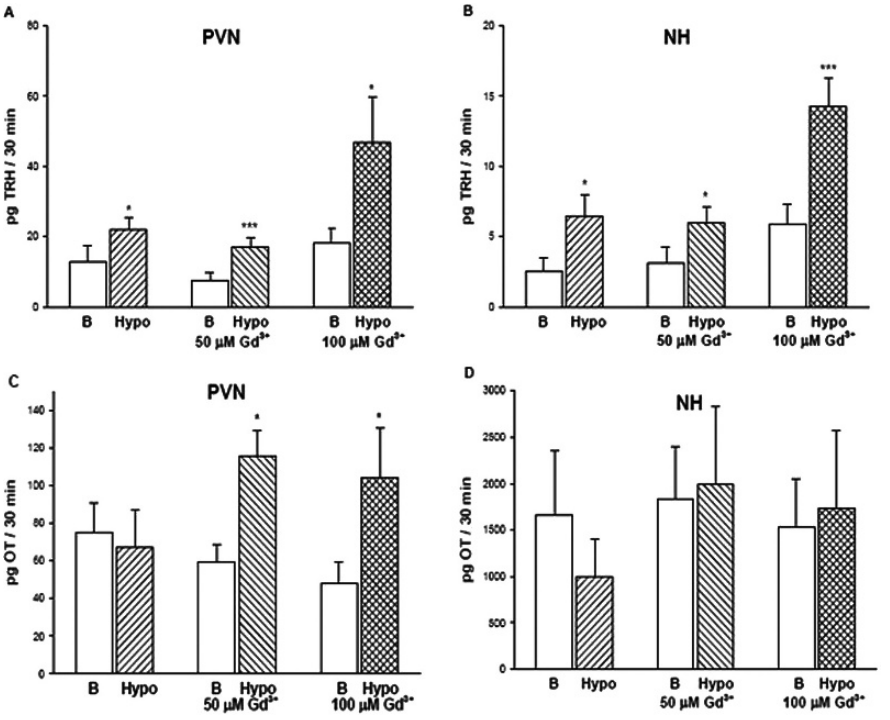

Fig. 4.2 Effect of 50 and 100 µM $GdCl_3$ on cell swelling induced (30% hyposmotic medium) TRH (upper panel) in PVN (**A**) and neurophypophysis (**B**) and oxytocin (OT, lower panel) secretion from paraventricular nucleus (PVN) and neurohypohysis (NH). Tissue explants were incubated for two subsequent 30 min periods in medium containing alternately basal and hyposmotic (hypo) medium. When effect of $GdCl_3$ was tested, it was present in both media. Data are shown as mean ± SE, n = 5. (*P<0.05, ***P<0.001). Adapted from Bacova et al. (2006b), with permission

Next part of the experiment was performed to ascertain whether the swelling-induced oxytocin secretion could be unmasked by the inhibition of the specific osmotic response. Gadolinium chloride is a potent blocker of mechanosensitive ionic channels (Yang and Sachs 1989, Oliet and Bourque 1996) and voltage-sensitive Ca^{2+} channels (Romano-Silva et al. 1994). Gadolinium blocks not only variety of mechanosensitive channels but also transient receptor potential (TRP) channels (for review Hamill 2006). In higher doses it also induces neurotransmitter release from the vesicular pool in rat brain synaptosomes (Lopatina et al. 2005). $GdCl_3$ and $La(NO_3)_2$ induced exocytosis and aspartate release are independent from extracellular Ca^{2+} and are resistant to lipid rafts disruption due to removing of the membrane cholesterol using cyclodextrin (Waseem et al. 2007). To exclude effect of gadolinium itself on secretion, the same dose (50 or 100 μM) of $GdCl_3$ was added into basal as well as stimulating (hypotonic) medium. The presence of $GdCl_3$ did not change hypotonicity-induced TRH release from PVN and NH (Fig. 4.2). In presence of lanthanide hypotonicity stimulated also oxytocin release from PVN (Fig. 4.2) suggesting that specific response of MNCs to osmolarity was inhibited and swelling induced exocytosis took place. Oxytocin secretion from NH remained unchanged.

The stretch-inhibited cation (SIC) channels of MNCs are highly permeable to Ca^{2+}, mediate significant Ca^{2+} entry and release of Ca^{2+} from internal stores (Zhang and Bourque 2006). To differentiate between possible mechanisms by which $GdCl_3$ is acting on swelling-induced oxytocin release (inhibition of mechanosensitive ionic channels or Ca^{2+} transport) we tested effect of hypotonicity and ethanol on oxytocin secretion from PVN, SON and NH at stringent Ca^{2+} free conditions. In part of experiments when $GdCl_3$ was present in the medium chelator EDTA was omitted to avoid lanthanide chelating.

Supraoptic nucleus: In Ca^{2+} depleted medium neither hypotonicity nor ethanol were able to induce oxytocin secretion from SON (Fig. 4.3A). When explants of SON were incubated in presence of 100 μM $GdCl_3$, secretory response to hypotonic medium appeared (Fig. 4.3B).

Paraventricular nucleus: While hypotonic medium in the presence of Ca^{2+} did not induce oxytocin secretion (Fig. 4.2) from the PVN, the absence of extracellular Ca^{2+} promoted oxytocin release from this structure in response to hyposmotic stimulus (Fig. 4.3C). $GdCl_3$ enabled both, hypotonicity and ethanol to have stimulatory effect on the release of oxytocin from PVN (Fig. 4.3D).

Neurohypophysis: Secretion of oxytocin from NH in response to hypotonic stimulation was not affected either by Ca^{2+} depletion (Fig. 4.3E) nor $GdCl_3$ treatment (Fig. 4.3F).

To exclude direct unspecific effect, the same conditions (except stimulus) were applied in basal and stimulated incubations (e.g. the same dose of $GdCl_3$ and/or Ca^{2+} depletion). Comparison of the amount of neuropeptides secreted in basal media with and without lanthanide did not reveal any effect of $GdCl_3$ on the TRH or oxytocin secretion. On the other hand, Ca^{2+} depletion seems to block specific response and unmask release of oxytocin from PVN in response

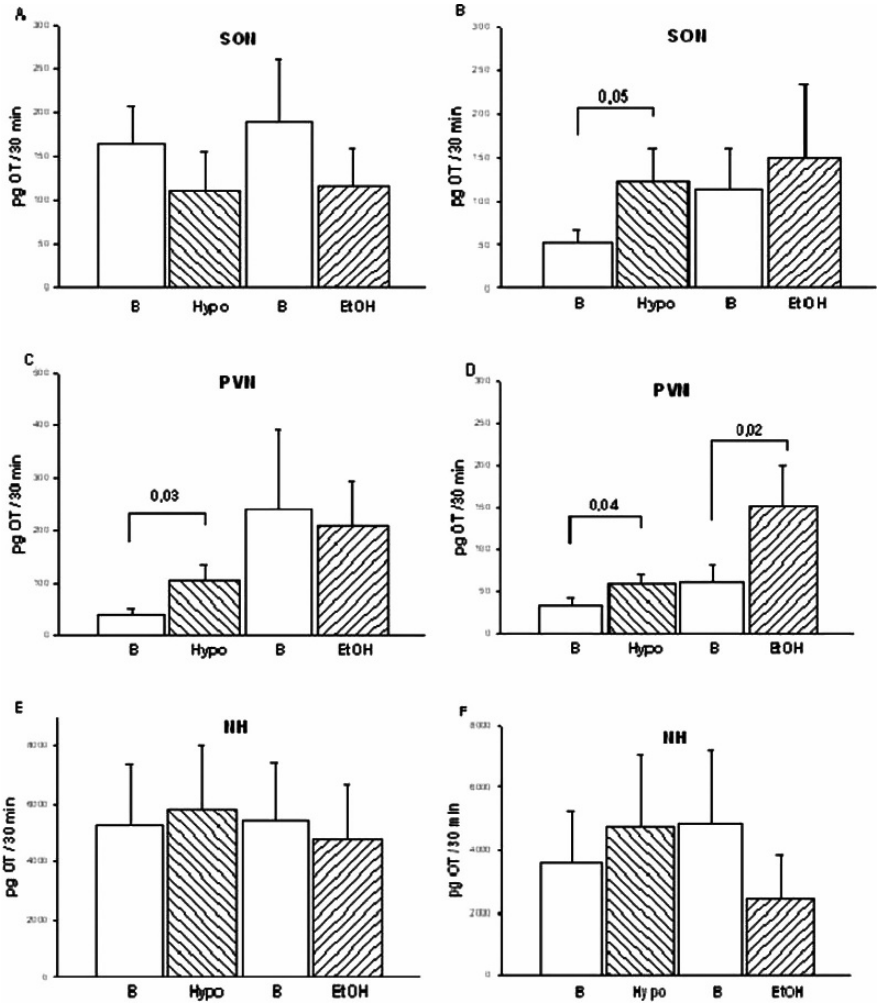

Fig. 4.3 Effect of Ca^{2+} free medium without (left panels) and with 100 μM $GdCl_3$ (right panels) on cell swelling induced oxytocin (OT) secretion. Swelling was induced by hypotonic (hypo) and ethanol (ETOH) containing isosmotic medium. Explants from SON (upper panels), PVN (middle panels) and from NH (lower panels) were incubated for four subsequent 30 min periods in medium containing alternately basal and stimulating medium (hyposmotic or ethanol). When effect of GdCl3 was tested, it was present in all media and EDTA was omitted to avoid chelating of Gd3 + . Data are shown as mean ± SE, n = 8–10. Adapted from Bacova et al. (2006b), with permission

to hypotonicity (Fig. 4.3C). Why we did not see this effect of Ca^{2+} depletion on oxytocin secretion from SON is unclear. We can only hypothesize that there may be an extra Ca^{2+}-depending factor or step involved in regulation of oxytocin secretion from PVN which might play only minor role in SON.

The presence of $GdCl_3$ in the medium resulted in an increased oxytocin secretion in the SON and the PVN to hypotonic and in the PVN also to ethanol containing medium. Therefore, it is likely that $GdCl_3$ in our experiments obviated a specific response to osmolarity changes and thus unmasked general non-specific cellular response to hypotonicity.

Mechanism by which lanthanide unmasks secretory response to hypotonicity is not clear. Thinnes et al. (2001) using a light-scattering approach on transformed normal or CF human B-lymphocytes in hypotonic Ringer solution found that the addition of 15–60 μM $GdCl_3$ increased cell swelling beyond normal values. Regulatory volume decrease (RVD) was not observed in those experiments. A corresponding effect was seen in isotonic Ringer containing $GdCl_3$. In both osmotic situations, Gd^{3+} – induced cell swelling was abolished by monoclonal mouse anti-human type-1 porin antibodies. Authors concluded that $GdCl_3$ opens plasmalemma-integrated porin channels and thus allows ions to follow their gradients resulting in cell swelling (Thinnes et al. 2001).

Gd^{3+} has been shown to have dose dependent dual effects on various transient receptor potential channels (TRP, for review Hamill 2006). Biological effects of TRP channels are typically mediated by Ca^{2+} influx. Two TRP channels that expressed in the brain are osmosensitive (for review see Orlov and Mongin 2007). TRPV1 (mechano/osmosensitive nonselective cation channel) is activated by cell shrinking and inactivated by cell swelling. TRPV4 expressed in neuronal population of subfornical organ and organum vasculosum laminae terminalis is activated by cell swelling and inactivated by cell shrinkage. The effect of relatively low concentrations of $GdCl_3$ at the level of PVN and SON in our experiments suggests that it could be TRPV-1 which was mediating specific response of magnocellular cells secreting oxytocin.

Osmosensitivity of magnocellular neurons in SON and PVN is strongly aided by adjacent glial cells (Orlov and Mongin 2007). Taurine release from glial cells is tonically regulated by physiologically relevant changes of cerebrospinal osmolarity. Extracellular osmolarity reductions as small as 7% increase tonic taurine release by more than 25% (Passantes- Morales et al. 1990, Deleuze et al. 1998). Once released, taurine potently inhibits electrical activity and the release of AVP in magnocellular neurons via neuronal glycine receptors (Hussy et al. 2000). Since gadolinium had no effect on the volume-sensitive release of taurine from glial cells and it seems also to be unrelated to Ca^{2+}-dependent transduction mechanisms (Pasantes-Morales et al. 1990) participation of this mechanism in the obviating specific mechanism and unmasking cell swelling-induced oxytocin secretion in our experiments is unlikely.

Gadolinium chloride is also a potent blocker of voltage-sensitive Ca^{2+} channels (Romano-Silva et al. 1994). Ca^{2+} free medium without an inhibitor was not able to promote swelling-induced oxytocin secretion from SON suggesting that the effect of $GdCl_3$ on Ca^{2+} transport was not the main mechanism of the Gd^{3+} effect. Whatever the mechanism is, our results showed that oxytocin is secreted from the PVN and the SON in response to swelling-inducing treatments after a blockade of specific response by $GdCl_3$.

Neither of treatments used was able to unmask swelling-induced oxytocin secretion from the NH. Neurohypophysis represents nerve terminals which can release peptides in response to swelling (Nikodemova et al. 1999). We have also shown that swelling is able to induce TRH but not oxytocin release from NH (Najvirtova et al. 2002, 2003). Depending upon the stimulus, oxytocin and vasopressin are released from the dendrites and axonal terminals of magnocellular neurons in either a coordinated or an independent manner. For example, suckling evokes oxytocin release in the SON before significant peripheral secretion occurs (Moos et al. 1989), whereas after osmotic stimulation, oxytocin and vasopressin release from the SON lags behind peripheral secretion (Ludwig et al. 1994). In oxytocin cells, electrical activity of cell bodies can result in the release of neuropeptides from nerve terminals in the posterior pituitary with little or no measurable release from the dendrites. Furthermore, some stimuli induce neuropeptide release from dendrites without increasing the electrical activity of the cell body, and without inducing secretion from oxytocin cell nerve terminals (Ludwig et al. 2002, 2005, Sabatier et al. 2003). It is therefore not surprising that the NH behaved differently than the SON and the PVN in response to stimulation and inhibition. Recently, Zhang et al. (2005) have shown an important role of neurophysin for vasopressin sorting in the regulated secretory pathway and its efficient packaging into large dense core vesicles. If this mechanism participates in different responses of the SON, PVN, and NH oxytocin secretion to hypotonic stimulation remains to be established.

4.6 The Physiological and Pathophysiological Significance

Cell swelling-induced peptide exocytosis is an ancient, essentially ubiquitous mechanism. Its physiological significance remains to be fully recognized.

Cell volume. Exocytosis of intravesicular material may help a cell to meet a relative extracellular hyposmotic challenge by expanding the plasmalemma. Minor osmotic changes undoubtedly occur frequently as a result of local or general homeostatic or metabolic processes.

Ischemia. When tissue becomes ischemic there is a shift to anaerobic glycolysis and production of metabolites which can result in an increase of intracellular osmolarity by 70 and 130 mOsmol/L after 15 and 60 min, respectively, thus increasing transmembrane osmotic pressure differences and producing cell swelling (Wright and Rees 1997). Peptides and proteins released after swelling could play an important role in the pathophysiology of ischemia and could be mediators of local or remote preconditioning when factors released from the ischemic tissue have protective effect against ischemia-reperfusion injury (Bartekova et al. 2004).

Disruption of specific osmosensitive mechanisms. Magnocellular neurons in the PVN and SON, specifically engaged in water and salt regulation, developed

their specific response to osmotic stimuli – release of vasopressin and oxytocin in response to hyperosmotic stimulation. This specific response could be obviated by $GdCl_3$ and at these conditions general secretory response to swelling-inducing stimuli emerged. This might be an explanation of the mechanism of the development of some clinical syndromes: Disruption of mechanosensitive gating in magnocellular neurosecretory cells perturbing specific response to osmotic stimuli could result in an inadequate secretory response of hormones engaged in water and salt regulation (e.g. stimulation of antidiuretic hormone secretion instead of inhibition in patients with hypervolaemic hyponatraemia). This kind of phenomenon could play a role at least in some types of the *syndrome of inappropriate secretion of antidiuretic hormone* SIADH. This syndrome (Miller 2006) often seen by clinicians, could be induced also experimentally in gerbils by brain infarction (Ejima et al. 2006). SIADH develops due to persistent detectable or elevated plasma AVP concentrations in the presence of continued fluid intake (Baylis 2003). Due to resulting inadequately high water reabsorption in kidney hypotonic hyponatraemia is connected with urine osmolality in excess of plasma osmolality. The major groups of causes of SIADH are: neoplasia, neurological diseases, lung diseases and a wide variety of drugs (Baylis 2003).

4.7 Conclusions

Swelling-induced peptide secretion represents important cellular reaction when in response to relative extracellular hypotonicity material stored in secretory vesicles is expulsed as a secretory burst of peptides and proteins from various types of cells. This type of secretory response might play an important role in pathogenesis of ischemia-induced changes. Swelling induces secretion from different parts of neuron: axons, dendrites and bodies. Magnocellular neurosecretory cells in the PVN and SON, engaged in water and salt regulation, developed specific response to osmotic stimuli – release of vasopressin and oxytocin in response to hyperosmotic stimulation. This specific response could be obviated by $GdCl_3$ and at these conditions general (unspecific) secretory response to swelling-inducing stimuli emerged. Pathological perturbation of the specific osmosensitive mechanism and emerging of unspecific (opposite) secretory response might be pathophysiological mechanism of the development of the *syndrome of inappropriate secretion of antidiuretic hormone* SIADH, characterised by stimulation of antidiuretic hormone secretion instead of inhibition in hypervolaemic patients with hyponatraemia.

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Chapter 5
Neuronal Mechanosensitivity in the Gastrointestinal Tract

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Abstract The ability to sense differing stimuli in the gut and translate this to alter a number of physiological outcomes is reliant on the mechanosensory and transductive capacity of the intestine. Mechanosensitivity is conferred by both extrinsic and intrinsic pathways in the gut and includes non-neuronal elements. This review focuses on the neuronal mechanosensitive elements within the GI tract and describes their functional and neuroanatomical features. The primary intrinsic neuronal mechanotransductive elements within the gut are intrinsic primary afferent neurons (IPANs) and contribute to the largely autonomous adaptation of the gut to intraluminal stimuli, allowing for peristalsis but also other reflexes such as secretion and local blood flow. The extrinsic mechanotransductive pathways run in vagal, pelvic and splanchnic circuits and can convey both innocuous and noxious aspects of the intestinal environment to the central nervous system including, but not limited to, mechanical stimuli. The exquisite ability of the gastrointestinal tract to sense different types of physical deformation and respond accordingly is conferred in part by the varying types of sensory mechanotransductive terminals. Such distinctions are exemplified by the purported roles of Intraganglionic Laminar Ending (IGLE) and Intramuscular Arrays (IMA) in mechanical sensing. Sensory mechanosensitive terminals can be found in all layers of the intestine where they subserve different stimulus modalities or varying degrees of stimuli such as distension or stretch. Intensive effort is currently being focused on the role of sensory neuronal mechanotransductive pathways in various 'hypersensitivity' disorders such as irritable bowel syndrome. An understanding of the physiological, pathophysiological and pharmacological distinctions between such neuronal mechanosensitive elements may bring substantive clinical pharmacotherapeutic benefit.

Keywords Intraganglionic laminar endings · intramuscular arrays · IPAN · irritable bowel syndrome · mechanoreceptors · visceral hypersensitivity

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A. Kamkin, I. Kiseleva (eds.), *Mechanosensitivity of the Nervous System*,

5.1 Introduction

The gastrointestinal tract possesses a substantial network of sensory neural circuitry that conveys information both within the gut and to the central nervous system. These systems allow for both the autonomous regulation of physiological processes such as peristalsis or local secretion and blood flow, in addition to providing for the central integration of information regarding the local environment within the gut. The following review looks at the anatomical features of neuronal mechanosensitivity in the gut by describing the gross features of innervation, followed by the most recent details that we currently know of cellular and molecular aspects of reception and transduction. This includes a discussion of mechanosensitive ion channels and continues by describing some of the known pharmacological properties of such mechanosensory nerves. It finishes with a discussion of the clinical relevance of targeting such nerves with novel pharmaco-therapies, by focusing on visceral afferent (sensory) function in inflammatory and functional bowel diseases.

5.2 Gastrointestinal Mechanosensory Pathways and Properties

5.2.1 Intrinsic Neurons

There are a number of cell types believed capable of sensory transduction and which utilise intrinsic sensory nerves in this process. For example, enterochromaffin cells are capable of secreting transmitters in response to mechanical or chemical luminal contents to activate motility or secretion in the gut (Cooke, 2000; Neal and Bornstein, 2007). Interstitial Cells of Cajal (ICC) and gastrointestinal smooth muscle contain mechanosensitive ion channels and thus are capable of eliciting a cellular 'response' to mechanical distortion or physical forces (Kraichely and Farrugia, 2007). All of these elements may work in concert with intrinsic nerves to ultimately project reflex changes in motility, blood flow or secretion in the gut well beyond a normal autocrine or paracrine process. This section of the review only focuses on the intrinsic neuronal elements in the GI tract.

Enteric afferent function is conferred by intrinsic primary afferent neurons (IPANs), which are activated by low threshold stretch or mechanical forces as well as conventional GI neurotransmitters, such as serotonin (Furness et al., 1998). They release transmitters capable of activating either downstream (anally-projecting) or upstream (orally-projecting) secretomotor, vasomotor or conventional motility pathways. IPANs perform this by propagating signaling to both ascending and descending interneurons via select neurotransmitters such as acetylcholine or CGRP (Bornstein et al., 2004; Vanner and Macnaughton, 2004). In regular peristalsis their function is critical, where a food bolus elicits a distension-sensitive activation of the IPAN allowing for

forward-projecting relaxation (accommodation) and retrograde contraction, allowing for bolus propulsion. In a physiological context it is believed that mucosal deformation from distension elicits transmitter release from the enterochromaffin (EC) cells, which in turn activates the IPAN, but it is possible that the IPAN can be activated independently from changes in mucosal shape or tension (Furness et al., 1998).

5.2.2 Extrinsic Neurons

The extrinsic pathways are conveyed via both vagal and spinal nerves. Vagal sensory nerves largely innervate the upper gastrointestinal tract and respond to a variety of physical and chemical stimuli. The nerve cell bodies are localized to the nodose ganglion before terminating in the nucleus tractus solitarius. Spinally projecting sensory pathways are comprised of splanchnic and pelvic nerves and are largely considered to variably innervate the entire GI tract. The cell bodies are generally found within the dorsal root ganglia and project to thalamic centres.

The functional degree of mechanical stimulation (physiological versus pathological) was traditionally viewed as being the main determinant of the gross neuroanatomical projection of mechanical (and other modalities) extrinsic sensory fibres from the viscera, where vagal sensory nerves largely convey innocuous stimuli such as physiological levels of distension, while recruitment of spinal sensory pathways was typically reserved for noxious or pain-evoking mechanical stimuli from the gut (Grundy, 2002). This view still pervades current thinking to some degree, where it is believed that most noxious mechanosensation is conveyed in spinal pathways simply because vagal projections are believed to be limited to the distal ileum, while painful sensation is received from the entire gastrointestinal tract. However, vagal afferent nerves can be traced to the colon, some of which at least are likely to be mechanoreceptive (Wang and Powley, 2000).

The termination of the receptive field of the afferent fibre within the layers of the gastrointestinal tract partly determines whether it is activated by stimuli most likely to be in the physiological or noxious range (Berthoud et al., 2004). Mechanoreceptors with receptive fields in close proximity to the gastrointestinal mucosa are likely to be directly acted upon by physical forces associated with luminal propulsion, as are many mechanoreceptive afferent fibres in the muscularis propria (Berthoud et al., 2004). Serosal mechanoreceptors, found in the outer layer of the gut, are more likely to be exposed to pathological forms of stretch or distension. It should be noted that all known types of mechanoreceptive afferent fibres can also respond to a variety of chemical stimuli, some of which may arise from normal hormonal and secretory processes associated with digestion and gastrointestinal motility (Ozaki et al., 1999; Page et al., 2002).

5.2.2.1 Vagal Mechanosensory Nerves

Neuroanatomical retrograde labeling has greatly enabled the anatomical identification of vagal afferent fibres, many of which are mechanoreceptive. Using such techniques, coupled with functional electrophysiological studies, has aided the delineation of form with function of such afferent fibres.

Mucosal afferent fibres were readily identifiable as possessing varicose endings that course and branch within the mucosa (Page et al., 2002). Mucosal afferent fibres are exquisitely sensitive to mucosal distortion in addition to possessing chemosensitivity (Page and Blackshaw, 1998), but may not respond to other forms of physical stimuli, such as muscle contraction or distension (Lynn and Blackshaw, 1999).

In the smooth muscle layers and myenteric plexus sandwiched between the muscle layers, two morphologically distinct classes of mechanoreceptor exist which are carried within the vagus nerve. These are termed intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMA) and are believed to sense different types of mechanical force (Powley and Phillips, 2002), although this view is not without contention. The IGLEs sit at the surface of the myenteric ganglia and it is believed this arrangement allows them the capacity to transduce shearing forces between muscle layers, acting as tension receptors (Lynn et al., 2003; Powley and Phillips, 2002; Zagorodnyuk et al., 2001). In contrast, the IMA runs within, and in parallel with, either circular or longitudinal smooth muscle layers, has a more specialized anatomical distribution in gastroduodenal and sphincter regions of the gut and is variably believed to be responsive to stretch and length changes in the muscle it innervates (Blackshaw et al., 2007; Powley and Phillips, 2002). In addition, the IMA is closely apposed to enteric glial cells and Interstitial Cells of Cajal (ICC), where it is believed their sensory function is dependent on their close proximity to these cell types. Indeed, ablation of ICC results in a loss of IMA function (Fox et al., 2002).

5.2.2.2 Spinal Mechanosensory Nerves

Spinal mechanosensitive afferents can be subdivided into splanchnic and pelvic afferents. They convey information from all layers of the gut including mucosal, muscle and serosal layers (Brierley et al., 2005a). Within these pathways, pelvic mechanosensitive afferent nerves are generally considered to afford a greater degree of sensation from graded distension in distal parts of the gut such as the colorectal region, compared to areas solely innervated by splanchnic nerves which have higher thresholds for sensory activation (Berthoud et al., 2004). Rectal IGLEs are believed to partly provide for this sensory gradation; they are functionally similar to vagal IGLEs and run in pelvic nerve pathways. Spinal sensory pathways also contain populations of nerves with their receptive fields on mesenteric and submucous blood vessels, which will respond to blunt probing (Brierley et al., 2004). As with vagal sensory nerves, spinal afferents can be polymodal (Ozaki and Gebhart, 2001) and display varying degrees

of chemosensitivity and differential pharmacological modulation (Brierley et al., 2005a,b).

5.3 Mechanosensitive Reception and Transduction: Molecular Features

As with the mechanosensory elements in somatic nerves and other cell types, either visceral or somatic, knowledge of the molecular basis of mechanosensation and mechanotransduction in the gastrointestinal tract is relatively poorly understood.

In all cell systems there are two types of mechanosensitive ion channels; those linked through the cytoskeleton and those linked solely through the membrane lipid bilayer (Gottlieb et al., 2004; Kraichely and Farrugia, 2007). In the gastrointestinal tract many non-neuronal cell types are believed to possess mechanosensitivity, such as enteroendocrine cells, enteric glia, Interstitial Cells of Cajal and the smooth muscle itself. This property is conferred at a molecular levels by any number of mechanosensitive ion channels found in the gut and include those affording calcium, sodium and potassium conductances (Kraichely and Farrugia, 2007; Lyford and Farrugia, 2003). Sensory neuronal ion channels may also possess mechanosensitivity, such as Kv1.4 expressed on sensory neurons projecting to the rat dorsal root ganglion (Binzen et al., 2006). The potassium channels comprise the largest class of mechanosensitive channels that alter gating in response to mechanical stimuli and include those of the Shaker family (Gu et al., 2001), two pore domain and large conductance potassium channels (Ghatta et al., 2006; Kraichely and Farrugia, 2007). As this review focuses on neuronal mechanosensitivity, we will restrict the discussion to intrinsic and extrinsic gastrointestinal neurons.

5.3.1 Intrinsic Primary Afferent Neuron (IPAN) Molecular Mechanosensitivity

The molecular basis of IPAN mechanosensitivity has not been extensively studied, partly because of difficulties in isolating the tissue environment in which to discretely probe IPAN mechanosensitivity and partly because of the limited selectivity of available drugs for stretch-activated ion channels. Contaminating influences from muscle contraction and other elements inexorably linked to the tissue connective framework contributes to this impediment (Kunze et al., 1999). Despite these limitations, it is known that a variety of stretch-activated ion channels or ligand-gated channels with mechanical sensitivity may contribute to activation (Raybould et al., 2004; Kunze et al., 1999). It has been demonstrated that gastrointestinal intrinsic primary afferent neurons directly respond to mechanical forces via stretch-activated channels, which are

gadolinium sensitive and blocked by charybdotoxin, an antagonist of large conductance (BK) potassium channels (Kunze et al., 2000). Overlayed on top of these direct mechanotransductive IPAN responses are the influences of a possible cocktail of endogenous transmitters, including purines (Cooke et al., 2004) and serotonin (Pan and Gershon, 2000), amongst others. These transmitter molecules are released from enterochromaffin cells (EC) or other enterocytes and capable of activating IPANs or modulating their activation (Raybould et al., 2004).

5.3.2 Extrinsic Neuronal Molecular Mechanosensitivity

In extrinsic gastrointestinal mechanoreceptors, in vitro studies have begun to flesh out the underlying molecular basis of mechanotransduction. In either vagal or rectal IGLEs, mechanotransduction appears to not involve synaptic chemical transmission and is benzamil-sensitive (Zagorodnyuk et al., 2003, 2005). Thus it is proposed that mechanical forces such as distortion or distension evoke stretch-activated channels directly on the IGLE itself to activate mechanotransduction. A number of caveats apply to these studies however, in that (*i*) it is difficult to dissociate the direct activation of the IGLE from activation secondary to changes in muscle contractile activity and (*ii*) the pharmacological agents used to inhibit mechanosensitive ion channels are not discerning in their selectivity (Hamill and McBride, 1996). Pharmacological blockade of muscle contraction with L-type calcium antagonists mostly accounts for (*i*), but until the selectivity issues are resolved, elucidating molecular loci of mechanotransduction on the basis of drug effect will remain difficult.

Also proposed to be gastrointestinal sensory neuronal mechanosensitive channels are the acid-sensitive ion channels (ASIC) of the epithelial sodium channel (ENaC)/degenerin (DEG) gene family (Kellenberger and Schild, 2002; Wemmie et al., 2006). ASIC null mice display altered mechanosensation from afferents in a range of regions of the gut (Page et al., 2005a) and the modulation of colonic mechanosensitivity by benzamil was dependent on the integrity of ASIC (Page et al., 2007a). Their expression and role in gastrointestinal sensation appears to differ from somatic sensory nerve expression and function and their encompassing mechanosensory role in spinal pathways has been questioned (Drew et al., 2004). Further research (and selective channel blockers) is therefore required to more clearly establish their role in visceral gastrointestinal mechanotransduction.

Transient receptor potential (TRP) channels also have mechanosensitive members (Ramsey et al., 2006). The most widely studied member of this family, TRPV-1, is found on umyelinated afferent C-fibres, many of which convey mechanosensitive signals in the gastrointestinal tract (Berthoud et al., 2001). While it is unlikely that TRPV-1 activation occurs directly in response to

mechanical force (Bielefeldt et al., 2006), channel desensitization via application of the TRPV-1 agonist capsaicin can result in diminished activation of vagal mechanoreceptors (Blackshaw et al., 2000). Tracing studies have localized the expression of a number of TRP channel subtypes to vagal ganglionic (nodose) cells with terminations in the upper gut (Zhang et al., 2004). It should be noted that TRP channels may also play an important role in ICC function (Kim et al., 2006), the integrity of which is pivotal to vagal IMA mechanoreception and transduction (Fox et al., 2002). TRP channels are also linked to DRG neurons through their association with Kv1.4 (Binzen et al., 2006), some of which at least may be visceral gastrointestinal mechanoreceptive neurons.

5.4 Pharmacological Modulation of Mechanosensory Nerves

Despite the limited number of ligands available to directly and selectively affect mechanosensitive ion channels, intrinsic and extrinsic mechanosensory nerves are directly sensitive to a substantial array of pharmacological agents (Blackshaw et al., 2007). Once again it should be noted that upstream and downstream neuronal and non-neuronal cells which may either recruit or involve IPANs in transmission can be pharmacologically modulated or have been shown to express a variety of receptors histochemically. These include of course smooth muscle, but also Interstitial Cells of Cajal, enteric glia and enterochromaffin cells (Patterson et al., 2001; Raybould, 2002; Ward and Sanders, 2006) (Kraichely and Farrugia, 2007; Ruhl, 2005). Downstream interneurons and motorneurons also release many different neurotransmitters such as VIP, PACAP, substance P, acetylcholine, nitric oxide and others (Bornstein et al., 2004; Christofi et al., 2004; Mann et al., 1999; Weber et al., 2001). Ligands acting at any of these receptors can experimentally modulate processes altering motility, secretion or local blood flow.

For intrinsic mechanosensory nerves, autocoids such as serotonin and purines have been the most extensively studied and act on the intrinsic afferent through a number of distinct receptor subtypes (Blackshaw et al., 2007; Raybould et al., 2004). These are mediators released primarily from enterochromaffin cells (Bertrand, 2003; Raybould, 2002). In addition, IPANs are sensitive to histamine, leukotrienes and a number of cytokines and prostanoids, which links IPAN activity with the immune system in the gastrointestinal tract (Clerc et al., 2002).

For extrinsic mechanosensory nerves there are some distinctions versus intrinsic afferent nerves as to the receptor populations they express, which may have relevance to selective pharmacotherapeutic targeting of mechanosensory afferent function. In particular it is believed that extrinsic afferent fibres express the transient receptor potential TRPV-1 receptor, upon which the agonist capsaicin acts. TRPV-1 is purported to be only sparsely present or absent on intrinsic neuronal populations in the gut (Ward et al., 2003).

Extrinsic mechanoreceptors are also modulated by bradykinin (Brierley et al., 2005b; Partosoedarso et al., 2001), galanin (Page et al., 2007b; Page et al., 2005b), γ-aminobutyric acid via $GABA_B$ receptors (Page and Blackshaw, 1999; Smid et al., 2001), purines (Brierley et al., 2005a; Page et al., 2002), metabotropic glutamate receptors (Page et al., 2005c; Young et al., 2007), ghrelin (Page et al., 2007c), protease-activated receptors (Kayssi et al., 2007), opioids (Ozaki et al., 2000) and serotonin (Hicks et al., 2002), to name a few. A more complete list of receptor couplings to visceral afferents has been reviewed elsewhere (Blackshaw et al., 2007). The potential for any of these targets to be used as drug strategies to modify extrinsic afferent function depends on their tissue, region and receptor subtype selectivity, as well as the pharmacotherapeutic indication for their use. This also does not include the potential loci for additional drug treatments targeting extrinsic afferent central termini or even second-order integrative centres, which may rightfully be considered therapeutically useful. An example of this are the cannabinoid ligands, which are effective in the treatment of abnormal gastroesophageal motility associated with gastroesophageal reflux disease without altering gastric mechanosensation (Lehmann et al., 2002).

5.5 Role of Mechanosensory Nerves in Gastrointestinal Diseases

Discerning the role of mechanosensory nerves in the pathology of gastrointestinal diseases is a complex task. The focus of this discussion relates to both inflammatory bowel diseases and functional bowel disorders, mainly because these are the predominant clinical end points being actively pursued in the context of drug discovery and development targeted towards visceral afferent nerves, with particular focus on the role of extrinsic afferent nerves in lower intestinal diseases.

Chronic relapsing and remitting inflammation of the intestinal tract is characteristic of ulcerative colitis (UC) and Crohn's disease (CD), collectively termed inflammatory bowel disease (IBD). Symptoms related to IBD include abdominal pain and discomfort, diarrhoea, bloating, changes in urgency, habit and sensation. These symptoms are, in whole or part, generated by the effects of inflammation on the functioning of sensory and motor pathways in the bowel that coordinate sensation and motility. In contrast, irritable bowel syndrome (IBS) is ostensibly defined on the basis of symptomatology, with the disorder stratified into diarrhea- or constipation-predominant IBS.

The effect of inflammation on the function of gastrointestinal visceral afferents is often pronounced, due to the influence of the many inflammatory mediators affecting visceral mechanosensitive (and chemosensitive) properties. However, in addition, inflammation or infection can affect either the structure, function, chemical or signaling coding of smooth muscle (Cao et al., 2006; Cao et al., 2004; Cook et al., 2000), enterochromaffin cells (Lomax et al., 2006;

Wheatcroft et al., 2005), glia (Nasser et al., 2007) and Interstitial Cells of Cajal (Altdorfer et al., 2002; Rumessen, 1996). It should also be pointed out that most of our understanding of cellular and molecular changes in afferent function in IBD comes from experimental animal models of colitis, in which there are few definitive models that adequately encapsulate clinical IBD, especially for Crohn's disease (Pizarro et al., 2003). Nonetheless, these models have given us at least some insight into how inflammation affects the neuro-immune axis generally, as well as gastrointestinal mechanosensory phenotype and function.

Chronic inflammation can significantly alter intrinsic neuronal cell properties (Lomax et al., 2005). Intrinsic sensory neurons become more sensitive to a wide range of stimuli (Lomax et al., 2005; Mawe et al., 2004; O'Hara et al., 2007). This section however will focus more on extrinsic sensory pathways, where the interest in modulation of visceral gastrointestinal nociceptive transmission as it relates to pain management associated with many gastrointestinal disorders is extensive, especially in functional disorders such as irritable bowel syndrome. In this sense the term visceral hypersensitivity is used to describe the lower thresholds for nociceptive recruitment. This description implies a primary disorder manifest in, or predominantly involving, extrinsic afferent nerves, but there may be an enhanced perception of such input also that attributes to the symptomatology of IBS (Mayer et al., 2008), in addition to the influence of solely intrinsic enteric elements (Lomax et al., 2005).

The search for mechanistic evidence for visceral afferent hypersensitivity in IBS is problematic, partly due to the limited extrapolation that can be made from current animal models, limitations in determining mechanisms from whole animal studies when using global endpoints and a lack of detailed knowledge on its pathophysiology (Mayer et al., 2008). This is most evident during any literature review of the basic science associated with IBS, where there is a preponderance of speculative review articles, with few comprehensive *in vitro* studies of sensory nerve function.

Those studies which have induced colitis with chemical or infective treatment have subsequently investigated visceral hyperalgesia, often using the visceromotor response in the whole animal as a marker of visceral hyperalgesia (Adam et al., 2006; Lamb et al., 2006; Larsson et al., 2006). A small number of studies have investigated colonic afferent properties directly following colonic inflammation and found an increase in spinal nerve sensitivity to colorectal distension (Beyak et al., 2004; Li et al., 2006; Olivar et al., 2000), even after partial recovery from colitis (Coldwell et al., 2007). Altered neurochemical markers associated with colitis include TTX-resistant sodium channels (Beyak et al., 2004), neurokinin NK-1 receptors (Palecek et al., 2003) and glutamatergic NMDA receptors (Li et al., 2006). A major focus of research has investigated the pharmacology of serotonin in IBS, with the advent of the first clinical treatments of IBS related to $5HT_4$ receptor agonism (Evans et al., 2007). 5HT can excite or inhibit spinal afferents as well as modulating intrinsic afferent and motor pathways (Greenwood-van Meerveld, 2007; Greenwood-Van Meerveld et al., 2006; Hicks et al., 2002; McLean et al., 2007). Purinergic drugs are also considered

pharmacotherapeutic candidates in visceral gastrointestinal hypersensitivity. There is some evidence of an role for P2X receptors in the hyper-excitability of spinal afferent mechanosensory nerves following experimental colitis (Galligan, 2004; Wynn et al., 2004).

There has been less research into pharmacological targeting of mechanosensory ion channels in gastrointestinal inflammation. Interestingly, increased colonic afferent discharge in response to tissue stretch following acute colitis was diminished in ASIC or TRPV-1 knockout mice (Jones et al., 2007). In addition, visceral hypersensitivity in response to colorectal distension in chemical colitis was reduced following blockade of TRPV-1 receptors in the rat (Winston et al., 2007). These results suggest that the vanilloid receptor plays a significant role in the development or maintenance of visceral hypersensitivity during, or after, experimental colitis.

The endocannabinoid system has recently emerged as a potential modulator of experimental colitis. Recent studies demonstrated that mice without cannabinoid CB_1 receptors developed a significantly greater form of experimental

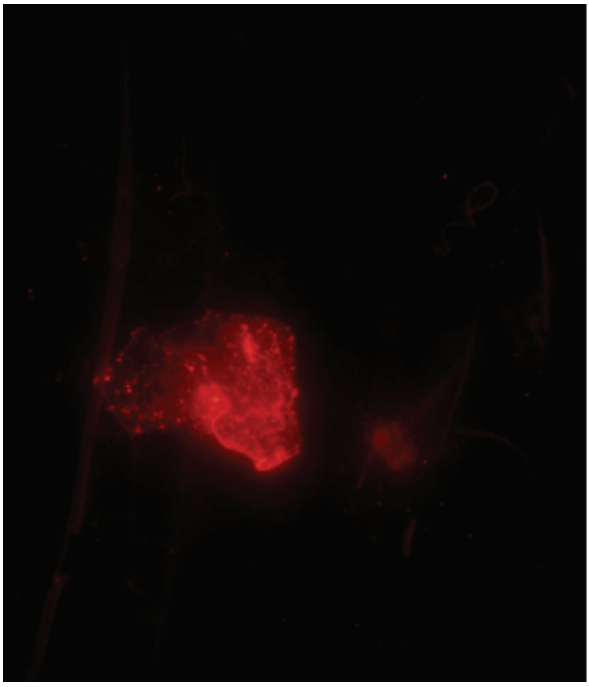

Fig. 5.1 Cannabinoid receptor labeling (CB_1 receptor immunoreactivity) in the human colon circular muscle layer. The fine mesh-like webbing was interspersed with densely labelled puncta. The morphology may be consistent with a sensory structure such as an intramuscular array, one of two types of terminal specialisations of extrinsic primary afferent neurones present in the gastrointestinal tract. Reproduced with permission from Hinds et al., (2006) Cannabinoid 1 CB1 receptors coupled to cholinergic motorneurones inhibit neurogenic circular muscle contractility in the human colon. Br J Pharmacol 148:191–199

colitis when compared to wild type animals (Massa et al., 2004), suggesting that the enteric cannabinoid system provides some degree of protection against the damage associated with gastrointestinal inflammation. Increased CB_1 receptor and anandamide amidohydrolase (FAAH) expression is also evidenced in a mouse model of experimental intestinal inflammation (Izzo et al., 2001), suggesting that the breakdown and turnover of endocannabinoids may be greater following inflammation. Importantly, cannabinoid agonists have been shown to attenuate visceral hypersensitivity in experimental colitis in rats (Sanson et al., 2006). Cannabinoids modulate enteric neurogenic and myogenic contractility in the human colon (Hinds et al., 2006; Smid et al., 2007) and there is preliminary evidence that cannabinoid CB_1 receptors are expressed on sensory structures in the human colon (Fig. 5.1). Moreover, the endogenous agonist for cannabinoid receptors, anandamide, also acts at TRPV-1 receptors (Di Marzo et al., 1998), providing a link between the endocannabinoid system and sensory C-fibres. The action of cannabinoid ligands at visceral sensory pathways is less certain, but CB_1 receptors have been localised to dorsal root ganglion cells, indicating that extrinsic afferent fibres express the CB_1 receptor (Hohmann and Herkenham, 1999). It is not know yet whether these are located on somatic or visceral afferents, or both (Hohmann, 2002).

5.6 Conclusions

In terms of realistic therapeutic targets for visceral hypersensitivity, any of the aforementioned receptors, cation channels or mechanosensitive channels may be candidates. There are also other pharmacological targets not covered in this review, but covered elsewhere (Holzer, 2001). Irrespective of the pathogenesis of inflammatory or functional bowel syndromes, to some extent all of the drug targets described earlier may modulate visceral hypersensitivity; but whether they all act on mechanosensitive sensory pathways is less likely and in addition, whether they provide any clinical relief or abrogation of symptoms may prove less predictable, as has been found for the limited effectiveness of $5HT_4$ ligands as IBS treatments (Mayer et al., 2008). What is certain is that as the animal models become more relevant and ligands of channel function more selective, the underlying basis behind neuronal mechanosensitivity in the gut will become clearer. Thus it will follow that its role in gut disease will also become more transparent. The ongoing quest to elucidate the molecular basis of mechanotransduction may yet yield up some novel targets to find their way to the clinic.

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Part II
Mechanosensitivity of the Receptors

Chapter 6
Mechanosensitivity of the Cochlea

Keiji Tabuchi and Akira Hara

Abstract Transient receptor potential (TRP) channels, widely expressed in both neuronal and non-neuronal cells, are activated by mechanical stimuli as well as by chemical and physical stimuli. The sense of hearing depends on the cochlea. Transduction channels at the tips of stereocilia of cochlear hair cells are the channels that convert mechanical sound stimuli to electrical action potential of the auditory nerve. Several types of TRP channels are expressed in the cochlea, which seem to be essential for maintaining the normal hearing. Despite extensive researches on the transduction channels, molecular identification of the mammalian transduction channel has not been achieved. In this review, we summarize the possible involvement of TRP channels in auditory mechanotransduction.

Keywords Transient receptor potential (TRP) channel · hearing · cochlea · transduction channel

6.1 Introduction

The generation of sound often originates in mechanical vibration, and sound is transferred by movement of air molecules in the direction of propagation (Yates, 1995). Movement of air molecules in the external auditory canal causes movement of the tympanic membrane, which in turn vibrates the middle ear ossicles and the cochlear fluid. The sense of hearing relies on the cochlea and its hair cells. The cochlea transduces rapid fluctuations in the atmospheric baseline pressure into a neural code of the auditory nerve. Sensory hair cells are the basic units that convert mechanical stimulation of sound into electrical signals of neurons. Transduction channels exist in the tip region of hair cells. They are mechanically-sensitive nonselective cation channels with a high permeability to calcium (Corey and Hudspeth, 1979; Ohmori, 1985; Jaramillo and Hudspeth,

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1991). Deflection of the stereocilia (the hair of hair cells) toward the tallest stereocilia leads to shearing of shorter stereocilia. The consequential increase of tension in tip links results in an influx of cations through the transduction channels to depolarize hair cells.

Sensorineural hearing loss is one of the most prevailing disabilities in humans. Estimates of the prevalence of moderate to severe bilateral sensorineural hearing loss fall 0.5–1 per 1000 live births. It has been further estimated that the prevalence may increase to 1.5–2 per 1000 children by age 6 years (Schneiderman, 1998).

The transient receptor potential (TRP) protein superfamily is a diverse group of voltage-independent cation-permeable channels. The ion channel was first described in Drosophila, and thereafter a lot of TRP channels have been identified. To date, about 70 TRP channels have been identified in genomes of humans, C. elegans and Drosophila. They have been categorized into 7 subfamilies by sequence homology: TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML (Liedtke and Kim, 2005). In invertebrate, TRPN1 (nomp C), Inactive (iav) and Nanchung (nan) are involved in transduction in bristle and chordotonal mechanoreceptors of Drosophila melanogaster (Gong et al., 2004; Walker et al., 2000, Kim et al., 2003). Several TRP channels have been reported to exist in the mammalian cochlea and considered to be candidates for the transduction channel of hair cells. TRP channels are attractive candidates for the mammalian cochlear transduction channel because they exhibit generally high conductance, low sensitivity and high calcium permeability (Owsianik et al., 2006; Corey 2006). In this review, we summarize the potential functional roles of TRP channels in auditory transduction of the mammalian cochlea.

6.2 TRPV Channels

The mRNAs of TRPV1, 2, 3 and 4 are detected in cochlear spiral ganglion cells (Kitahara et al., 2005). Several studies focused on altered responses to mechanical stimuli in *trpv*$1^{-/-}$ mice. TRPV1 knockout mice displayed altered responses of the bladder and the jejunum to mechanical stretch (Birder et al., 2002; Rong et al., 2004). An immunohistochemical study revealed the presence of TRPV1 in hair cells, supporting cells of the organ of Corti and spiral ganglion cells (Takumida et al., 2005). Although those findings suggest the possible response of TRPV1 to mechanical stimuli in the cochlea, there has been no report on mechanotransduction of cochlear hair cells by TRPV1. The functional role of TRPV1 in the hair cells has not been clarified so far. However, TRPV1 expressed in the spiral ganglion cells is suggested to have a role in gating spontaneous and evoked auditory nerve excitability (Zhou et al., 2006).

TRPV4 was found by screening sequence tag databases for sequences with similarity to TPPV1, TRPV2 and the Caenorhabditis elegans TRPV isoform

Osm-9. TRPV4 is a nonselective cation channel and was originally identified as an osmotically activated channel (Liedtke et al., 2000; Strotmann et al., 2000). An in situ hybridization study has revealed TRPV4 mRNA expression in hair cells, spiral ganglion and stria vascularis in the cochlea (Liedtke et al., 2000). In fruitflies, the TRPV4 orthologue, Nanchung, and its heteromeric partner, Inactive, are expressed in chordotonal neurons and are essential for hearing (Gong et al., 2004; Kim et al., 2003).

We examined effects of disruption of TRPV4 in mice on cochlear functions by analyzing auditory brainstem response (ABR) and distortion-product otoacoustic emission (DPOAE) (Tabuchi et al., 2005). DPOAE is supposed to be closely related to an active process in the organ of Corti and is a useful test of outer hair cell function (Whitehead et al., 1992). TRPV4-deficient mice (*trpv4*$^{-/-}$) were produced and maintained on a C57BL/6 background (Mizuno et al., 2003). Cochlear functions were evaluated in the TRPV4-deficient mice as well as the wild mice at 2 or 6 months of age. Although there was no significant difference in the ABR thresholds between these animals at 2 months of age, ABR thresholds were elevated in TRPV4-deficient mice at 6 months of age. This finding demonstrated that TRPV4 was not required for the normal development of the cochlea but that disruption of TRPV4 caused late-onset hearing loss in mice. Because there was no significant difference in DPOAE between these animals at 2 or 6 months of age, it seems that elevation of ABR thresholds observed in TRPV4 knockout mice was not caused by dysfunction of hair cells.

We also examined the effects of TRPV4 disruption on the vulnerability of the cochlea to acoustic injury (Tabuchi et al., 2005). The ABR threshold shifts were larger in the TRPV4-deficient mice than in the wild mice one week after the acoustic overexposure of 128 dB SPL, suggesting that TRPV4-deficient mice are vulnerable for acoustic trauma.

The hearing impairment is described as nonsyndromic when it is not associated with other clinical manifestations. Autosomal dominant nonsyndromic hearing loss (DFNA) typically exhibits delayed onset, and the hearing is progressively impaired with age (Van Camp et al., 1997). Greene et al. (2001) have reported that DFNA25, an autosomal dominant nonsyndromic hearing loss, maps to 12q21–24 and that *TRPV4* is one of candidate genes in this region. They also reported that hearing loss was often late-onset and progressive in DFNA25 in human. It is therefore possible that mutations in human *TRPV4* may account for some forms of hereditary hearing loss.

Based on the cochlear fluid, the cochlea is divided into three compartments, i.e. the endolymphatic, the perilymphatic and the interstrial spaces, which were filled with extracellular fluid. Because TRPV4 is expressed in the marginal cells of the stria vascularis (Liedtke et al., 2000), it is possible that TRPV4 may play important roles in detecting the mechanical or osmotic stimuli of endolymph (extracellular fluid in the endolymphatic space) and in maintaining the inner ear fluid.

6.3 TRPA1 Channel

TRPA1 was originally identified as a protein overexpressed in liposarcoma cell lines (Jaquemar et al., 1999). TRPA1 is unique among mammalian TRPs in having as many as 17 ankyrin repeats (Kwan et al., 2006). Corey et al. (2004) screened all TRPs of the mouse for expression in the inner ear using in situ hybridization. They found TRPA1 mRNA in vestibular hair cells firstly at embryonic day 17 when the hair cells became mechanically sensitive (Corey 2004; Geleoc and Holt, 2003). Immunohistochemically, TRPA1 was stained at the tips of the stereocilia in vestibular hair cells of the bullfrog and the mouse (Corey et al., 2004). Based on these findings, TRPA1 initially appeared to be a strong candidate for the transduction channel of hair cells.

Despite the attractive findings of TRPA1 in transduction of the hair cells as mentioned above, recent findings hardly support the hypothesis that TRPA1 is the transduction channel. Firstly, the expressions of TRPA1 in the hair cells of the cochlea and the vestibular system were weak in mice (Corey et al., 2004). Secondly, hair cell transduction was not affected by allyl isothiocyanate, a known activator of TRPA1 (Jordt et al., 2004; Corey et al., 2006). Finally and definitely, two independently generated TRPA1 knockout mice did not show any hearing loss (Kwan et al., 2006; Bautista et al., 2006). The TRPA1 knockout mice exhibited normal ABR and normal transduction currents in vestibular hair cells. Kwan et al. (2006) concluded based on their findings that TRPA1 is not essential for hair-cell transduction but contributes to the transduction of mechanical, cold, and chemical stimuli in nociceptor sensory neurons.

6.4 TRPML3 Channel

The identification of mutations in the human mucolipin (*trpml1*) gene in mucolipidosis type IV led to the initial description of the TRPML subfamily (Bach, 2005). The mammalian TRPML subfamily now consists of TRPML1, 2 and 3.

The classical semidominant mouse mutant varitint-waddler (Va) exhibits early-onset hearing loss, vestibular deficit (circulating behavior, imbalance and waddling), pigmentation abnormalities, and perinatal lethality. A second allele, Va^J, which arose in a cross segregating for Va, shows a less severe phenotype (Di Palma et al., 2002). The mutated gene encodes TRPML3 in both alleles (Di Palma et al., 2002). Histological studies of $+/Va$ heterozygotes revealed several abnormalities in the cochlea. The tectorial membrane in the heterozygotes is thicker than that in normal ears. The cell density in the spiral ganglion was decreased in the heterozygotes (Deol, 1954). Cochlear hair cells and supporting cells made an undifferentiated cell mass as early as P11. In addition, nuclear and cytoplasmic irregularities were noted in the marginal cell layer of the stria vascularis (Atiba-Davies and Noben-Trauth, 2007).

Although TRPML3 cannot be ruled out as a transduction channel candidate, studies in cultured cells have shown that the TRPML3 channel exists primarily in cytoplasmic organelles and not in the plasma membrane (Venkatachalam et al., 2006; Grimm et al., 2007).

6.5 Conclusion

Hair cells are mechanosensitive cells, which are essential for transduction of sound stimuli. Transduction channels exist in the tip region of the steleocilia. Several features of the mammalian transduction channel have been reported. The channel is a nonselective cation channel which is permeable to all the alkali cations and to many divalent cations. The channel exhibits high calcium permeability. Unquestionably, molecular identification of the transduction channel is required to fully understand the mechanotransduction of sound stimuli. Although the mammalian transduction channel still remains mystery, TRP channels are now the most attractive candidates. In addition, cochlea contains several fluid compartments, and hair cells are surrounded by extracellular fluid of the cochlea. Because TRP channels mediate responses for osmotic and chemical stimuli as well as those for mechanical stimuli, additional works will shed light on the specific roles of TRP channels in physiology and pathology of the cochlea.

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Chapter 7
Osmoreceptors in Cochlear Outer Hair Cells

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Abstract The mammalian outer hair cells (OHCs) are mechanical effectors of the cochlea. The slow motility of OHCs is considered to be a possible adaptive mechanism in the cochlea. It is suggested that passive calcium-independent slow motility induced by hyposmotic activation may have physiological or pathological significance even in normal or impaired hearing of cochlear origin. The cell swelling induced by hyposmotic stimulation has been shown to be accompanied by an increase of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in OHCs. This $[Ca^{2+}]_i$ increase may subsequently activate metabolic processes including phosphorylation in OHCs. Therefore, the ionic environment and the changes in osmolarity of the inner ear may affect the OHC motility, thereby varying the sensitivity of the inner ear to the sound. The functional expression of transient receptor potential vanilloid 4 (TRPV4) is involved in the hypotonic stimulation-induced Ca^{2+} influx in OHCs. It is suggested that TRPV4 may function as an osmo- and mechanosensory receptor in OHCs. Recent study showed that hyposmotic stimulation can induce nitric oxide (NO) production by the $[Ca^{2+}]_i$ increase, which is presumably mediated by the activation of TRPV4 in OHCs. NO conversely inhibits the Ca^{2+} response via the NO-cGMP-PKG pathway by a feedback mechanism. Any disturbance in the homeostasis of inner ear fluids may therefore affect the functional properties of OHCs by NO via the activation of TRPV4, thereby influencing the delivery of auditory information. In this review, volume regulation in OHCs also will be discussed.

Keywords Osmosensory receptor · volume regulation · cochlea · outer hair cell · hypotonic stimulation · nitric oxide · transient receptor potential vanilloid 4

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7.1 Introduction

Inner hair cells (IHCs) and outer hair cells (OHCs) are the two sensory receptors of the mammalian cochlea, and exhibit innervation and functional properties. The OHCs are innervated by large myelinated efferents and are exhibit somatic motility (Dallos, 1992; He et al., 2004) that is thought to contribute to the enhancement of cochlear frequency selectivity by generating negative damping (Dallos and Corey, 1991), although recent work also suggests that the hair bundle generates forces that could be involved in this process (Robles and Ruggero, 2001). The IHCs appear to perform a more passive role and are innervated by the majority of afferent auditory nerve fibers, hence giving rise to the major neural output of the cochlea.

The mammalian OHCs are mechanical effectors of the cochlea. The somatic motility of OHCs is divided into a fast and a slow motility (Zenner et al., 1985; Brownell et al., 1985; Zenner, 1986; Ashmore, 1987). The fast OHCs motility is metabolically sensitive and able to operate at high frequency without the involvement of acto-myosin or other enzymatic molecules of the cytoskeleton (Brownell et al., 1985; Ashmore, 1987). This fast motility is believed to provide mechanical amplification on a cycle-by-cycle basis which produces sharpening of tuning and decreased threshold during cochlear sensory-neural transduction. On the other hand, the slow motility was considered to be a possible adaptive mechanism in the cochlea (Zenner, 1986). The slow motility in OHCs may function to shift the operating point of the basilar membrane but probably contribute little to the mechanical amplification of the cochlea (Zenner et al., 1985; Zenner, 1986). Slow motile responses can be divided into an active calcium-dependent motility and passive calcium-independent shape changes (Dulon and Schacht, 1992). The calcium-dependent slow motility is based on activation of actin-myosin systems (Zenner, 1986; Dulon et al., 1990; Kalinec et al., 2000). In contrast, osmotic effect induced by hyposmotic stimulation causes a slow shape change which is accompanied by a cell swelling independent of calcium and actin-myosin systems (Dulon et al., 1987, 1988).

Previous study showed that the cell swelling induced by hyposmotic stimulation was accompanied by an increase in the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in OHCs (Harada et al., 1994) as well as other types of cells (Hazama and Okada, 1988; Weskamp et al., 2000; Okada et al., 1990; Suzuki et al., 1990; McCarty and O'Neil, 1991). Therefore, the osmotic swelling-induced $[Ca^{2+}]_i$ increase may subsequently activate metabolic processes including phosphorylation in OHCs, which results in affecting the OHC motility. There have also been several previous studies on the osmotic properties of OHCs in relation to the fast OHC motility (Jerry et al., 1995; Oghalai et al., 2000). Brownell and his colleagues suggested that the electromotility might be a product of an electro-osmotic mechanism (Jerry et al., 1995). Previous study also showed that hyposmotic stimulation results in magnitude and gain increase of the fast motility (Sziklai and Dallos, 1997). Those studies suggested that the ionic environment

and the changes in osmolarity of the inner ear may affect both fast and slow OHC motility, thereby varying the sensitivity of the inner ear to sound.

Any disturbance in the homeostasis of inner ear fluids may thus affect the functional properties of OHCs, thereby causing a disorder in the delivery auditory information in the auditory signaling transduction.

Osmoresponsive neurons are found in many parts of the CNS (Bourque et al., 1994). They contribute to fluid balance in the body. Osmoreceptors sense change in osmotic pressure. Osmosensory transduction is considered to be triggered specifically by changes in the volume of the neuron, and not by changes in ionic strength. The ability of cells to regulate their volume if exposed to an anisotonic environment is a fundamental physiological function. The ability of cells to regulate their volume is essential for maintenance of cellular homeostasis under anisotonic environmental conditions. To maintain both function and volume relatively undisturbed, cells have developed mechanisms that sense and oppose volume changes.

In this review, I will discuss the role of Ca^{2+}, nitric oxide and TRPV4 on the hyposmotic stimulation-induced slow motility of outer hair cells.

7.2 Signal Transduction for Osmoreception in Outer Hair Cells

The swelling of a cell can be observed when it is exposed to a hypotonic solution with subsequent changes of osmolarity. At the cellular level, an osmotic stimulus can be understood as a mechanical stimulus, because an osmotic cell swelling stretches the cell membrane, a mechanical force parallel to this membrane. This membrane stretch caused by cell swelling might be the first signal in response to hypo-osmotic stress. The first mechanosensitive processes may function as backup mechanisms for cell protection (Hamill and Martinac, 2001). Regulatory volume decrease (RVD) represents an immediate and local response to minimize the effects of altering osmotic equilibrium during hyposmotic stimulation (Lang et al., 1998a).

The physical force causes direct stretch of ion channels and surface receptors through alteration of membrane rigidity, fluidity and composition. Intracellular Ca^{2+} has been shown to play a key role during cell volume regulation in a number of cell types (Hazama and Okada, 1988; Weskamp et al., 2000; Okada et al., 1990; Suzuki et al., 1990; McCarty and O'Neil, 1991; Tinel et al., 2000; Foskett et al., 1994).

Hyposmotic swelling elicits an increase in $[Ca^{2+}]_i$ with the Ca^{2+} influx from the extracellular spaces and/or the Ca^{2+} release from internal stores in many types of cells (Lang et al., 1998a). The ubiquity of the hyposmotic cell swelling-induced $[Ca^{2+}]_i$ increase makes Ca^{2+} an important candidate for a role as osmotransducing signal. It has been reported that hyposmotic stimulation results in a $[Ca^{2+}]_i$ increase, which was accompanied by a cell shortening and swelling in isolated OHCs (Harada et al., 1994; Surin et al., 2000). An increase

in $[Ca^{2+}]_i$ largely depends on the extracellular Ca^{2+}, not the Ca^{2+} release from internal stores in OHCs (Harada et al., 1994; Surin et al., 2000). The stretching of the cell membrane by hyposmotic stimulation causes a Ca^{2+} influx through stretch-activated channel in several types of cells (Christensen, 1987; Okada et al., 1990). The stretch-activated channels with permeability to Ca^{2+} in the lateral cell membrane of OHCs have been characterized (Ding et al., 1991; Iwasa et al., 1991). A stretch-activated channel blocker, Gd^{3+} inhibited the hyposmotic stimulation-induced $[Ca^{2+}]_i$ increase in OHCs (Harada et al., 1993a). Therefore, hyposmotic cell swelling opens the stretch-activated non-specific cation channels, which allows a Ca^{2+} influx and results in the $[Ca^{2+}]_i$ increase in OHCs as well as other types of cells.

Previous studies have shown that the membrane potential was hyperpolarized by hyposmotic cell swelling in several types of cells (Welling and O'Neil, 1990; Kawahara et al., 1991, Dube et al., 1990; Hazama and Okada, 1988). It has been suggested that the hyposmotic cell swelling-induced hyperpolarization is due to an intracellular K^+ efflux through Ca^{2+}-activated K^+ channels (Taniguchi and Guggio, 1989; Ubl et al., 1988; Dube et al., 1990).

In OHCs, the hyposmotic cell swelling-induced $[Ca^{2+}]_i$ increase leads to an activation of Ca^{2+} -activated K^+ channels at basolateral membrane of OHCs which results finally in a reversible hyperpolarization of OHCs by K^+ efflux by means of whole-cell patch clamp technique (Fig. 7.1; Harada et al., 1993a; Iwasa et al., 1991) and the K^+-sensitive dye PBFI (Takeda-Nakazawa et al., 2007). These results provided clear evidence that the decrease of $[K^+]_i$ during hyposmotic stimulation may correlate with the hyperpolarization of OHCs. Osmotic cell swelling has been reported to be associated with the activation of Ca^{2+}-activated K^+ channels, which play a role in volume regulation in several types of cells (Christensen, 1987; Hoffman et al., 1984; Ling et al., 1992; Park

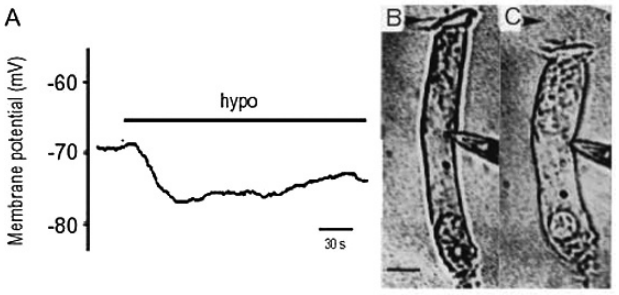

Fig. 7.1 Hyperpolarization of OHCs induced by hyposmotic stimulation. (**A**) Changes of membrane potential under current clamp mode (clamped at zero current). Hyposmotic stimulation induced membrane hyperpolarization from the resting potential of –69 to –76 mV in OHCs. (**B,C**) This hyperpolarization was accompanied by a cell swelling during hyposmotic stimulation in (**A**). Scale bar is 10 μm. Reproduced from (Takeda-Nakazawa et al., 2007) with permission from Elsevier

et al., 1994; Taniguchi and Guggio, 1989; Ubl et al., 1988; Weiss and Lang, 1988; Weskamp et al., 2000; Hazama and Okada, 1988; Vázquez et al., 2001).

There are three types of Ca^{2+}-activated K^+ channels: big conductance (BK_{ca}) , intermediate conductance (IK_{ca}) and small conductance (SK_{ca}). Previous studies have shown the evidence that IK_{ca} and SK_{ca} channels are also activated and contribute to volume regulation during RVD although BK_{ca} channel is the major K^+ conductive pathway during RVD in several types of cells (Hafting et al., 2006; Sheader et al., 2001; Jorgensen et al., 2003; Vázquez et al., 2001; Wang et al., 2003a; Köhler et al., 1996). In OHCs, the hyperpolarization of OHCs during hyposmotic stimulation was inhibited by quinine and Ba^{2+}, a blocker of Ca^{2+}-activated K^+ channels (Harada et al., 1993a). However, these agents are significantly less selective in their actions and may affect multiple different K^+ channels. Previous studies have shown that OHCs may posses BK_{ca} and SK_{ca} channels (Spreadbury et al., 2004; Housley and Ashmore, 1992; Skinner et al., 2003; Dulon et al., 1998; Oliver et al., 2000; Kong et al., 2006) although IK_{ca} channels are poorly characterized and have not yet been demonstrated in the cochlea. The types of K_{ca} channels for volume regulation in OHCs thus need to be further investigated.

Previous studies have shown that direct acoustic and mechanical stimulation to the lateral OHC wall to causes length changes (Brundin et al., 1989; Brundin and Russell, 1994; Canlon and Brundin, 1991; Chan and Ulfendahl, 1999). Such reports suggested that the selectivity and sensitivity of the cochlea may result in the sharply tuned mechanical response expressed by the OHCs during sound stimulation. Hyperpolarization of the OHCs contributes motility, which is followed by an elongation of the cellular body (Ashmore, 1987; Santos-Sacchi and Dilger, 1988; Zenner et al., 1987) in contrast to other non-sensory cells. It is speculated that the mechanical response of the OHCs to actively amplify the travelling wave is largely dependent on the membrane potential rather than the transmembrane current (Santos-Sacchi and Dilger, 1988). It seems to be likely that a stretch of the lateral membarane in the OHCs is physiological during sound stimulation. Under pathophysiological conditions, any changes of the inner ear fluid volume or composition can also possibly provoke such the stretch of lateral OHC membrane and hyperpolarization with the well-known clinical findings of tinnitus, hearing loss and vertigo, respectively (Horner, 1991; Zenner and Ernst, 1993; Zenner et al., 1994). Osmotic stress also causes biochemical signals through functional regulation of membrane proteins, which is mediated through the protein phosphorylation and dephosphorylation. The functional role of biochemical signals during volume regulation in OHCs remains to be elucidated.

7.3 Role of Nitric Oxide

Nitric oxide (NO), a gaseous membrane permeate messenger, has been thought to play an important role in signaling processing as a neurotransmitter or a neuromodulator in the olfactory system (Collmann et al., 2004), visual system

(Wang et al., 2003b) and central neural system (Esplugues, 2002). NO is synthesized by NO synthase (NOS). In the peripheral auditory organ, Ca^{2+}-dependent constitutive NOS, the neuronal NOS (nNOS) and the endothelial NOS (eNOS) have been demonstrated by means of immunocytochemistry (Franz et al., 1996; Gosepath et al., 1997; Heinrich et al., 2004). Those studies showed that nNOS and eNOS were localized in several types of cells in the cochlea, such as IHCs, OHCs, spiral ganglion neurons, and supporting cells (Franz et al., 1996; Gosepath et al., 1997; Heinrich et al., 2004). Recent studies using the NO-sensitive dye DAF-2 showed NO production to occur in several types of cells in the guinea pig cochlea (Shen et al., 2003, 2005; Takumida and Anniko, 2001; Yukawa et al., 2005; Shen et al., 2006b; Shi et al., 2001; Takeda-Nakazawa et al., 2007). Therefore, NO may play an important role in the inner ear function (Takumida and Anniko, 2002).

Previous studies showed that hyposmotic stimulation induced nitric oxide production in endothelial cells (Kimura et al., 2000, 2004). They suggested that NO may play an important role in regulating volume regulation and mechanical stress to the cell membrane in those cells. In OHCs, simultaneous measurement of cell length and NO production showed that hyposmotic-induced rapid cell swelling preceded NO production (Fig. 7.2; Takeda-Nakazawa et al., 2007). The hyposmotic stimulation-induced NO production was abolished in the absence of extracellular Ca^{2+} while cell swelling was still observed in these OHCs. The Ca^{2+} influx by hyposmotic-induced cell swelling thus causes NO production. It is suggested that a Ca^{2+} influx was the only preferential source for stimulating the NO production by hyposmotic stimulation in OHCs (Takeda-Nakazawa et al., 2007) as previously reported in other types of cells (Dedkova and Blatter, 2002; Lin et al., 2000; Mizuno et al., 2000; Li et al., 2003; Wang et al., 1996). Previous studies suggest that the hyposmotic cell swelling induces the ATP release, which results in NO production by autocrine effects in endothelial cells (Kimura et al., 2000). In contrast, suramin, a P2 receptor antagonist did not inhibit the hyposmotic stimulation-induced NO production in OHCs whereas Gd^{3+}, a stretch-activated channel blocker inhibited it (Takeda-Nakazawa et al., 2007). Thus, the hyposmotic-induced cell swelling may cause membrane stretch and this permits the Ca^{2+} influx. In turn, the $[Ca^{2+}]_i$ increase results in NO production in OHCs.

There is increasing evidence that NO also influences the mechanism of cellular Ca^{2+} homeostasis in several cell types by either a positive or negative feedback mechanism (Clementi and Meldolesi, 1997; Wang and Robinson, 1997). It has been suggested that the effects of NO are involved in the NO-cGMP-PKG signaling pathway in the sensory system of olfaction (Breer and Shepherd, 1993) and vision (Cudeiro and Rivadulla, 1999). Morphological studies have shown the NO-cGMP-PKG pathway to exist in the cochlea (Michel et al., 1999; Takumida et al., 2000). Hyposmotic stimulation-induced NO inhibits the $[Ca^{2+}]_i$ increase by a negative feedback mechanism in OHCs (Takeda-Nakazawa et al., 2007). This inhibitory effect of NO on the hyposmotic stimulation-induced Ca^{2+} signaling were mediated by the NO-cGMP-PKG signaling pathway (Takeda-Nakazawa et al., 2007).

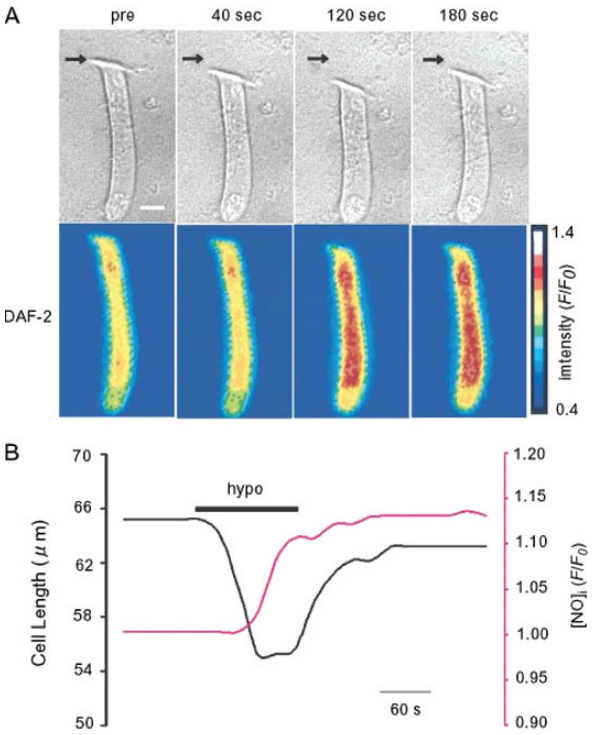

Fig. 7.2 Simultaneous measurement of cell length and NO production during hyposmotic stimulation in OHCs. (**A**) Bright field images of cell shortening and pseudo-color displays of DAF-2 fluorescence signals in an isolated OHC during hyposmotic stimulation (250 mOsm). The images of bright field and DAF-2 were taken at the times indicated above each pair of images. Note that the cell shortening was observed at 40 sec after onset of hyposmotic stimulation whereas no significant change in DAF-2 fluorescence was observed in this cell. An irreversible increase in DAF-2 fluorescence was observed accompanied with the cell swelling. Scale bar is 10 μm. (**B**) Time course of changes in cell length and NO production in A. The DAF-2 fluorescence intensities are normalized relative to the initial value ($F/F_{0;}$ right vertical axis) and are plotted in red; changes in cell length are shown on the left vertical axis and are plotted in black. The horizontal bar at the top of the trace indicates the timing of hyposmotic stimulation. Reproduced from (Takeda-Nakazawa et al., 2007) with permission from Elsevier

NO has been suggested to modulate the Ca^{2+}-activated K^+ channels by an inhibitory and enhancement effect in neurons (Zsombok et al., 2000), pituitary nerve terminals (Ahern et al., 1999) and brain (Shin et al., 1997). The inhibitory effect of NO on the hyposmotic stimulation-induced the Ca^{2+} response also leads to the inhibition of the hyposmotic stimulation-induced K^+ efflux and $[K^+]_i$ decrease (Takeda-Nakazawa et al., 2007). It cannot be ruled out the possibility that NO may directly inhibit the hyposmotic stimulation-induced K^+ efflux. Taken together, NO in cochlear OHCs may play an important role in

compensating the changes in the osomolarity in the inner ear fluids and volume changes in the perilymphatic and endolymphatic spaces.

Both nNOS and eNOS, Ca^{2+}-dependent NOS isoforms, are likely to contribute to the NOS signal in OHCs since these two isoforms are present in OHCs (Gosepath et al., 1997).

Recent study directly provided morphological evidence suggesting the possible interaction between nNOS and $P2X_2$ receptor in the apical region of OHCs (Shen et al., 2006b). However, 7-NI, a potent and selective inhibitor for nNOS, did not inhibit the both the hyposmotic stimulation-induced Ca^{2+} response and NO production (unpublished data). As for nNOS/eNOS double staining, eNOS immunofluorescence labeling was apparent in the basolateral membrane in the OHCs whereas the labeling for nNOS was mainly restricted to the apical region of OHCs and absent in the basolateral membrane in the OHCs (unpublished data). These findings indicate that that the main isoform of NOS on the hyposmotic stimulation-induced NO may be eNOS rather than nNOS. Heinrich et al. reported that the quantitative immuno-electron microscopic analysis revealed cellular differences in the degree of eNOS and nNOS expression in OHCs (Heinrich et al., 2000). They suggested that these differences might be connected to the different subcellular binding properties of nNOS and eNOS. They concluded that these two constitutive NOS (nNOS and eNOS) might be located at different subcellular sites and might be regulated by the different Ca^{2+} response. Therefore, the different role of eNOS and nNOS may also be associated with different cellular upstream and downstream signaling molecules, which respond to different extracellular signals and are responsible for different cellular functions in OHCs. However, it cannot be excluded the possibility that nNOS may also affect the hyposmotic stimulation-induced NO production and Ca^{2+} response in OHCs. Further study will be needed to detect the functional role of eNOS in isolated OHCs.

7.4 Functional Coupling of TRPV4

Transient receptor potential vanilloid 4 (TRPV4), the Ca^{2+}-permeable channel, has been recently proposed as an osmo- and mechanosensitive channel (Liedtke et al., 2000; Strotmann et al., 2000; Suzuki et al., 2003). In addition to hypotonic stimulation, the TRPV4 channel also responds to heat (Guler et al., 2002), phorbol derivatives (Watanabe et al., 2002), low pH (Suzuki et al., 2003), and several endogenous ligands (Watanabe et al., 2003), indicative of a multifunction of TRPV4 in cells.

In the mouse and guinea pig inner ear, the expression of TRPV4 has been detected in both the OHCs and the IHCs, as well as in spiral ganglion neurons (Liedtke et al., 2000; Takumida et al., 2005; Shen et al., 2006a; Takeda-Nakazawa et al., 2007) and is supposed to play a role in inner ear function. TRPV4 mRNA and protein were expressed in IHCs, OHCs, and spiral ganglion

neurons based on the findings of RT-PCR, single-cell RT-PCR, and immunohistochemistry, whereas they were negative in TRPV4$^{-/-}$ mice cochleae (Shen et al., 2006a).

Functional expression of TRPV4 is involved in the hypotonic stimulation-induced Ca^{2+} influx in OHCs of the mouse cochlea (Shen et al., 2006a). Therefore, TRPV4 may be a competent receptor responsible for hypotonic stimulation in OHCs and may function as an osmo- and mechanosensory receptor in OHCs. 4α-phorbol 12,13-didecanoate (4α-PDD), a specific activator for TRPV4 (Watanabe et al., 2002) can induce a $[Ca^{2+}]_i$ increase by the Ca^{2+} influx in OHCs whereas 4α-PDD failed to induce a Ca^{2+} response in the TRPV4$^{-/-}$ OHCs (Shen et al., 2006a). Ruthenium red is known to be an inhibitor of TRPV4 (Watanabe et al., 2002). When the wild-type OHCs were pre-incubated with 10 μM ruthenium red in the presence of extracellular Ca^{2+}, the hypotonic stimulation and 4α-PDD-induced the increase in $[Ca^{2+}]_i$ was inhibited (Shen et al., 2006a). It has been shown that immunoreactivity for TRPV4 was apparently expressed in the soma of OHCs (Fig. 7.3; Shen et al., 2006a; Takeda-Nakazawa et al., 2007). TRPV4 may have a coupling from the cell surface in the lateral plasma membrane of OHCs to a functional role for the intracellular TRPV4 pool and affect somatic motility of OHCs. Therefore, TRPV4 may play an important role in the active function of OHCs in the cochlear micromechanics. Both the hyposmotic stimulation-induced NO production and Ca^{2+} response was blocked by Gd^{3+}. It is concluded that the hyposmotic-induced cell swelling may increase membrane tension and this activates TRPV4 which permits the Ca^{2+} influx. In turn, the $[Ca^{2+}]_i$ increase results in NO production in OHCs.

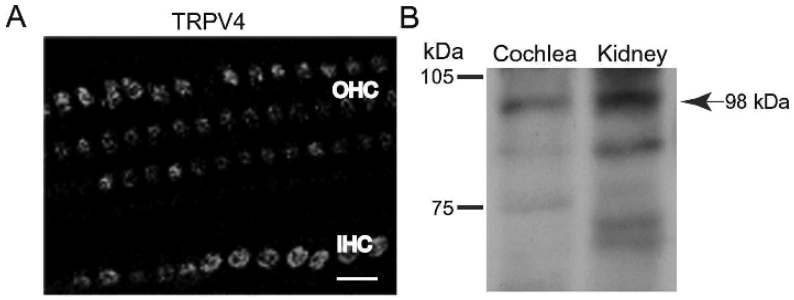

Fig. 7.3 Expression of TRPV4 protein in the guinea pig cochlea. (**A**) Immunofluorescent staining of TRPV4 expression in the guinea pig cochlea with TRPV4 antibody. Expression of TRPV4 was apparent at the soma of OHCs and IHCs. Scale bar, 20 μm. (**B**) TRPV4 expression was also analyzed by western blotting in cochleae extracts of guinea pigs. Kidney extracts were used as a positive control. Western blot bands for TRPV4 (98 KDa) were demonstrated in both cochlea and kidney (arrow). Negative control was performed by incubating TRPV4 antibody with the control antigen. Reproduced from (Takeda-Nakazawa et al., 2007) with permission from Elsevier

Although hypotonic solution can activate stretch activated channels directly through membrane stretch, recent evidence suggests that signaling intermediaries during TRPV4 activation are involved. TRPV4 channel activity is not increased in direct response to pressure-induced membrane stretch (Strotmann et al., 2000). Osmotic stretch has been shown to activate phospholipase A_2 (PLA_2), which hydrolyzes and releases arachidonic acid (AA) from membrane phospholipids (Vriens et al., 2004). Downstream metabolites of AA appear to activate TRPV4 because blocking PLA_2/AA signaling or downstream metabolites prevents activation by hypotonic solutions (Vriens et al., 2004). Recent study showed the possible role of tyrosine kinase-dependent phosphorylation in OHCs (Raybould et al., 2007). However, information on the OHCs function of tyrosine kinase-dependent phosphorylation is not fully available. The role of tyrosine kinase-dependent phosphorylation during TRPV4 activation in OHCs still remained to be obscured. The functional significance and role of tyrosine kinase-dependent phosphorylation in OHCs should therefore be explored. The accumulated data, including the results in $TRPV4^{-/-}$ mice, also suggest that pressure and shear stress directly activate TRPV4, which needs direct coupling of the cell membrane (Suzuki et al., 2003; O'Neil and Heller, 2005). These two osmotic response patterns, mechanical stimulation of the cell membrane and activation of intracellular phosphatase/kinase signalling cascades, need to be further investigated for the role of TRPV4 on osmosensory transduction pathway in OHCs.

It is interesting to note that any disturbance in the homeostasis of inner ear fluids may therefore affect the functional properties of OHCs via TRPV4, thereby causing a disorder in the delivery auditory information in the efferent auditory signaling transduction.

7.5 Volume Regulation

The ability of cells to regulate their volume if exposed to an anisotonic environment is a fundamental physiological function (Lang et al., 1998a). The volume regulatory mechanisms play a role in various cellular functions including epithelial transport, metabolism, excitation, migration, cell proliferation and apoptosis (Nilius et al., 1995; Okada et al., 2001; Lang et al., 2000, 1998b). Under hypotonic conditions, the influx of water along its osmotic gradient leads to cell swelling. Various types of cells are characterized by an initial swelling which is, however, followed by a volume decrease despite continued exposure to the hypotonic solution (Grinstein et al., 1982; Hazama and Okada, 1988; Hoffman et al., 1984; Welling and O'Neil, 1990; Lang et al., 1998a,b; Okada et al., 2001).

It has been suggested that this so-called regulatory volume decrease (RVD) following the initial cell swelling results from the loss of K^+ and Cl^- associated, in turn, with a loss of intracellular water molecules (Okada et al., 2001). The

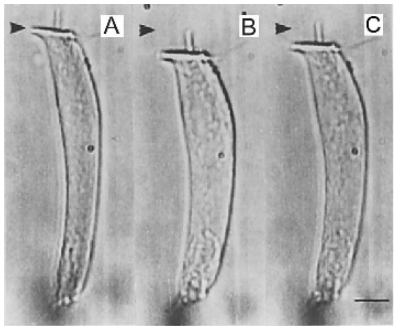

Fig. 7.4 Regulatory volume decrease in OHCs during hypomotic stimulation. (**A**) OHCs before hyposmotic stimulation. (**B,C**) OHCs during hyposmotic stimulation. The elongation of OHCs accompanied by volume decrease was observed in this cell in the continued presence of hypotonic solution. Scale bar is 10 μm

activation of volume-regulatory K^+ efflux has been shown to be controlled by the $[Ca^{2+}]_i$ increase (Hazama and Okada, 1988; Christensen, 1987; McCarty and O'Neil, 1991; Suzuki et al., 1990). The K^+ efflux activated during RVD is largely based on a Ca^{2+}-activated K^+ channel in several types (Ubl, et al., 1988; Taniguchi and Guggio, 1989). It has been reported that RVD was also seen in OHCs (Harada et al., 1994, 1993b; Belantseva et al., 2000). Figure 7.4 shows RVD in OHCs during hyposmotic stimulation. As previously reported, exposure to hypo-buffer was accompanied in parallel by a decrease in the cell length (cell shortening) and an increase in the cell volume (cell swelling) in OHCs (Fig. 7.4: Dulon et al., 1987; Harada et al., 1994). The increase in cell length indicates a decrease in the cell volume (Dulon et al., 1987; Harada et al., 1994). Therefore, a decrease and an increase in the cell length during hyposmotic stimulation indicates an increase and a decrease in the cell volume, respectively, as shown previously (Dulon et al., 1987; Harada et al., 1994).

Both the increase in $[Ca^{2+}]_i$ and RVD in OHCs during hyposmotic stimulation depended on the availability of extracellular Ca^{2+} and a Gd^{3+}-sensitive Ca^{2+} response (Harada et al., 1993a,b, 1994). Recent studies showed that TRPV4 is essential for Ca^{2+} influx and onset of RVD (Becker et al., 2005; Arniges et al., 2004). They suggested that TRPV4 would be a likely candidate for mediating RVD (Becker et al., 2005; Arniges et al., 2004). A previous study showed that direct perfusion of hypotonic solution into the perilymphatic spaces affects the summating potential and the compound action potential (Klis and Smoorenburg, 1994). Salt (2004) also showed that volume changes of endolymphatic spaces induced by nontraumatic low-frequency tones affect cochlear function. These effects were reversible. Under any disturbance in the homeostasis of the inner ear fluids, volume regulatory mechanisms in OHCs may play an important role in compensating for volume and pressure overload. The underlying volume-sensing mechanisms that enable OHCs to undergo

RVD and to limit this process until a certain volume is reached are not yet fully understood.

7.6 Conclusion

In contrast to non-sensory cells, hyperpolarization induced by the membrane stretch during osmotic swelling subsequently may induce the OHCs somatic motility, which results in an elongation of the cell body. The membrane stretch by direct acoustic and mechanical stimulation to the lateral OHC wall cause the OHC length changes. The resulting OHC length could tune the basilar membrane by bringing about a shift in its mean position. Thereby, the responsiveness of the cochlear partition is improved. It seems likely that the membrane stretch of the lateral OHC wall is physiological events during sound stimulation. Therefore, the hyperpolarization induced by membrane stretch may participate to the control of the OHC motility and fine tuning under physiological conditions, respectively. Changes of osmolarity in the inner ear fluids have been considered to cause some clinical disorders such as tinnitus, fluctuating hearing loss, and Meniere's disease. Therefore, the existence of volume regulatory mechanisms in OHCs may contribute to compensate for volume and pressure changes under pathophysiological conditions.

The ability of OHCs to produce NO in considerable amounts might be of clinical importance in situations associated with the physiological and pathophysiological conditions. The role of NO in the regulation of the OHC function still remains to be elucidated, but experiments with several reagents such as blockers for NOS and TRPV4 in cochlear dysfunction would be of great interest, both in terms of the pathology of inner ear disease and of the development of more specific therapies for dysfunction of the auditory signaling pathway.

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Chapter 8
Are Stretch-Activated Channels an Ocular Barometer?

James C. H. Tan and Minas T. Coroneo

Abstract Cells are subject to mechanical forces within their environment. This chapter reviews the concept of mechanosensitivity as it pertains to stretch-activated channels and the possibility that they have barometric functions within the eye. The relevance of these concepts to the pressure-related eye disease of glaucoma is discussed.

Keywords Stretch-activated channels · mechanically-gated channels · mechanosensitivity · pressure · glaucoma · optic nerve · retinal ganglion cells · trabecular meshwork · cytoskeleton

8.1 Introduction

The ocular biomechanics facilitating vision are orchestrated by a complex interplay between cells and their immediate environment within the tissues of the eye. The eye has an outer coat of connective tissue that helps it maintain its shape and dimensions and protect its contents (Fig. 8.1). The connective tissue coat must be tough enough to withstand various mechanical forces: extraocular muscle contractility, eyelid blinking, shearing and resistance within the orbit and ocular trauma. It must balance this toughness with allowing neurovascular elements to penetrate its substance, selective fluid and solute permeability, and maintaining transparency in its parts for the uninterrupted passage of light.

The eye's contents within its outer coat are kept under pressure, called the intraocular pressure. This intraocular pressure helps maintain the shape of the eye, relative positions of the refractive apparatus and photoreceptors, and influences ocular perfusion pressure. Intraocular pressure is regulated

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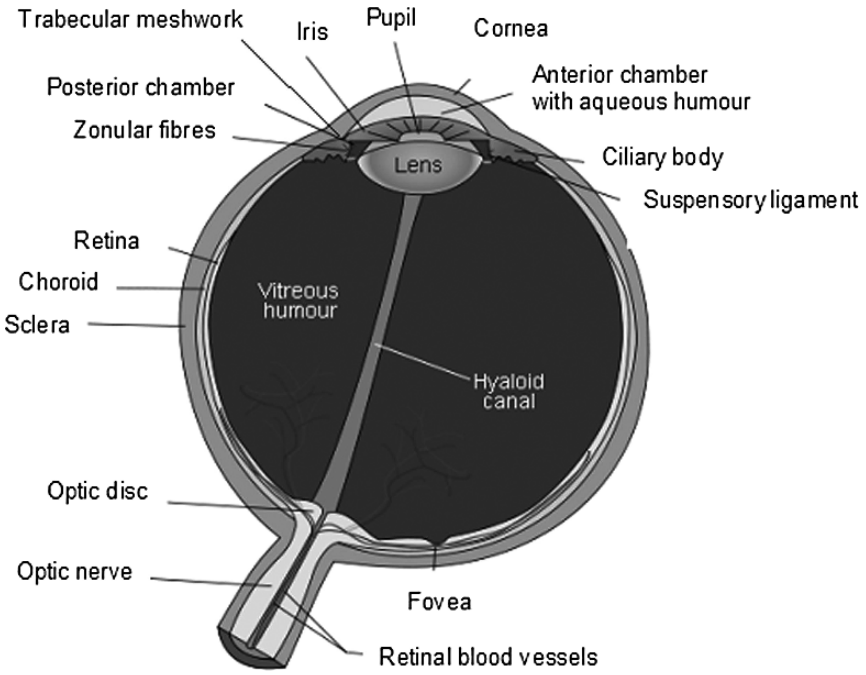

Fig. 8.1 The eye has a tough outer connective tissue coat comprising the cornea and sclera. The cornea, lens, zonular fibers and ciliary body make up the refractive and focusing apparatus of the eye. Aqueous humour dynamics and intraocular pressure are regulated by the ciliary body and trabecular meshwork. Together the iris, trabecular meshwork, ciliary body and choroid form the uveal tract. Photoreceptors and retinal ganglion cells are part of the layer of the retina. Cells in the cornea, sclera, conjunctiva, lens and uveal tract have been found to have mechanosensitive potential

by complex mechanisms within the ciliary body and trabecular meshwork affecting secretion and drainage of the aqueous humour. The contractile ciliary body further forms a dynamic biomechanical unit with the crystalline lens to focus light.

Cells or sensory elements that respond to mechanical stimulation have been identified in the cornea, sclera, conjunctiva, lens and uveal tract (Belmonte et al., 1997; Cooper et al., 1986; Mintenig et al., 1995). These cells are subject to mechanical forces externally which must be sensed, responded and adapted to—hydrostatic pressure, tension, compression, torsion, vibration, shear and stretch that can deform the cell. There are also internal forces to be responded to such as osmotic changes and swelling. If mechanical stresses are excessive or not adequately responded to, they may damage cells, and cause cell death and disease.

When intraocular pressure becomes too high acutely it affects the normal functioning of ocular tissues, causing corneal oedema, iris ischaemia, lens

opacity, trabecular meshwork alteration, and affecting the retinal circulation. Less dramatic elevation of intraocular pressure is not benign however as it is linked to retinal ganglion cell death, altered neuronal axoplasmic flow, availability of neurotrophic factors, deformation of the optic nerve's lamina cribrosa connective tissue, retinal astroglial changes and generation of toxic metabolites.

For cells to be 'mechanosensitive' they must be able to respond to physical forces acting within their immediate world. The sensing of these forces and conversion into signals that promote a response is termed 'mechanotransduction'. Much fundamental research has sought to define the cellular mechanisms mediating mechanosensitivity. One such mechanism implicates the coupled and interconnected cytoskeleton-adhesion-extracellular matrix complex, which has critical roles in signal transduction (Fig. 8.2) (Bershadsky et al., 2003). Another mechanism concerns membrane-bound mechanically-gated channels that are sensitive, speedy in response, and allow huge ion influxes and amplified signals (Hamill and Martinac, 2001). This chapter aims to review the putative role of stretch-activated membrane channels (SACs) in mechanosensitivity in the eye and the pressure-related disease of glaucoma.

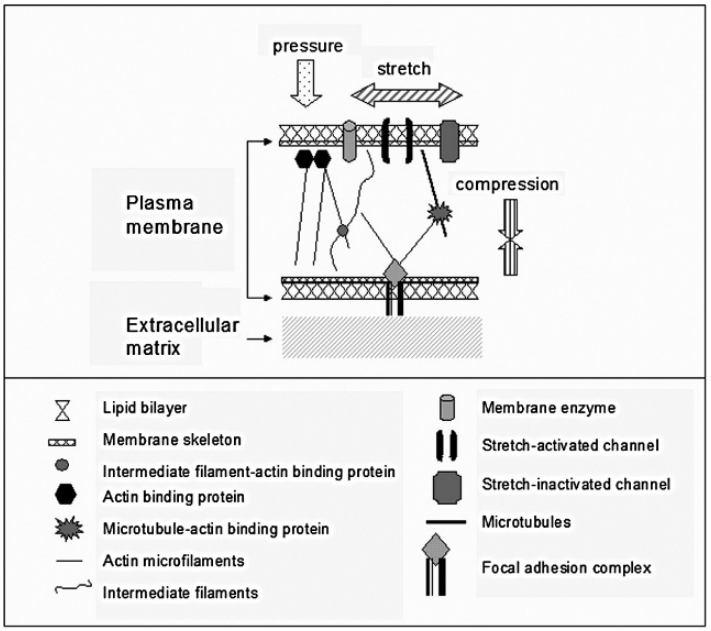

Fig. 8.2 Diagram illustrating the cellular machinery undergirding mechanosensitivity. Mechanically-gated channels and the cytoskeleton-adhesion-extracellular matrix complex are interlinked systems which allow the cell to sense and respond to mechanical forces such as stretch, pressure and compression

8.2 Stretch-Activated Channels

8.2.1 The Concept of Mechanosensitive Ion Channels and Their Discovery

Patch clamping has provided critical insights into the pathophysiology of diseases in which ionic channel abnormality is implicated, such as cystic fibrosis, epilepsy and Lambert-Eatons disease (the "channelopathies"). Mechanically-gated channels or SACs are ionic channels gated to respond to mechanical forces acting at the plasma membrane. They open when the cell membrane receives physical stimuli such as stretch, shear, and displacement.

SACs were first conceived in whole cell studies of specialised mechanosensory neurons (Katz, 1950; Loewenstein, 1959; Morris, 1990). They were subsequently identified by Guharay and Sachs (1984) who noticed that channel activity increased when they applied suction while patch clamping cultured chick skeletal muscle. Almost simultaneously Brehm et al. (1984) reported a similar phenomenon in embryonic xenopus muscle. Using patch clamping Guharay and Sachs (1984, 1985) and Sachs (1987) found that with SACs: (1) activation increased with the square root of applied pressure with channels opening more frequently as suction increased; (2) opening probability increased with higher K^+ concentrations, and (3) these channels showed selective cation permeability with greatest conductance for potassium, then caesium, sodium and lithium, suggesting a large aqueous pore. Their results support the notion that cells respond to applied pressure in a definable way and reproducibly. SACs are now thought to explain in part the relationship between cellular stress and reproducible cell excitation through a single conformation change of a channel. Membrane patch-clamping studies need to be interpreted with the knowledge that the suction used in the technique may itself alter membrane geometry and the properties of membrane proteins, and that channel properties can appear very different in whole cell studies (Zhang and Hamill, 2000).

Since their original description, different types of SACs have been identified across various cell types and organisms. Organisms range from prokaryotes such as bacteria and archea that possess cell walls, to eukaryotic cells such as unicellular yeasts and multicellular animal cells that lack cell walls but possess a cytoskeleton. They have been identified in many mammalian organs, including the central and peripheral nervous system and the eye.

8.2.2 Mechanical Gating and Ionic Selectivity

The most distinctive property of SACs is that their gating is dependent on membrane tension. The nature of the gating and ionic selectivity varies between organisms, as seen in the difference between prokaryotes, such as bacteria, and eukaryotes.

Bacteria depend on rigid cell walls to protect against excessive swelling and deformation, although this theoretically renders them less sensitive to mechanical stimuli. How bacteria might respond to external forces has been suggested by the discovery and cloning of mechanically-gated channels in Escherichia coli (Martinac et al., 1987; Sukharev et al., 1994, 1997). These have also been demonstrated in other bacteria, both Gram positive and negative which have different cell wall structures (Blount and Moe, 1999; Zoratti et al., 1990; Zoratti and Petronilli, 1988). A class of E coli receptors called MscL are postulated to protect the bacterium from osmotic damage as they open just below pressures that would otherwise disrupt the bacterial cell membrane. The SACs of prokaryotes are generally activated by high tensions, have high ionic conductance, and lack ionic specificity when compared with those of eukaryotes. It is known however that genetically mutating prokaryotic channels can confer upon them the gating characteristics of eukaryotic channels (Hamill and Martinac, 2001).

Eukaryotic cells lack rigid walls but their plasma membranes are internally supported by an elaborate cytoskeleton, as illustrated in Fig. 8.2. The cytoskeleton provides internal scaffolding for linking or tethering various proteins such as signalling molecules and mechanosensitive channels. Disrupting actin increases the conductivity of mechanosensitive channels (Wan et al., 1999). SACs appear to be cation selective, being permeable to cations such as K^+, and divalent cations such as Ca^{2+} (Hamill and Martinac, 2001; Morris, 1990). K^+ is the predominant intracellular cation having a key role in maintaining the resting potential of cells, with its intracellular concentration approximately 140 mEq/L. Transmembrane K^+ movements are physiological but excessive K^+ efflux may serve as a trigger for apoptosis, as is seen early in apoptosis prior to fragmentation. K^+-selective SACs have been identified in the trabecular meshwork of the eye (Gasull et al., 2003). Ca^{2+} functions as a second messenger following mechanical perturbation, affecting signal transduction and exocytosis (Bi et al., 1995; Steinhardt et al., 1994; Terasaki et al., 1997).

A more recently identified family of channels with weak inwardly-rectifying K^+ conductance includes TRAAK and TREK-1 and TREK-2, which are found widely in the central nervous system and also cardiovascular tissue, where its role as a baroreceptor has been postulated (Lansman et al., 1987; Lesage et al., 1996; Maingret et al., 1999; Patel et al., 1998, 2001; Terrenoire et al., 2001). In vitro, TREK-1 is sensitive to suction, osmotic swelling and shrinkage; TRAAK is sensitive to suction and pressure; both are activated by arachidonic acid, acidosis and inhalational anaesthetics. In the retina they have been found in ganglion cells, amacrine, horizontal and rod bipolar cells, and outer segments of photoreceptors (Fink et al., 1998; Reyes et al., 2000).

Mammalian corneal epithelium, non-pigmented ciliary epithelium, and trabecular meshwork cells have volume-regulated outwardly-rectifying chloride channels which respond to hypotonic cellular swelling and potentially have roles in maintaining the clarity of the ocular media and secretion and outflow of the aqueous humour (Comes et al., 2005; Mitchell et al., 2002; Soto et al., 2004; Srinivas et al., 2004).

8.2.3 Pressure Gating of Stretch-Activated Channels

Patch-clamping studies show that negative pressures of 200 mmHg are high enough to rupture cell membranes. Most SACs are maximally activated at suction pressures of 10–100 mmHg (Cooper et al., 1986). Evidence for the cytoprotective role of SACs includes experiments in E.Coli in which SACs of the MscL channel type open at pressures just under levels that would disrupt membranes (Martinac et al., 1987; Sukharev et al., 1994, 1997).

In some cells possessing K^+-selective SACs, stretch-inactivated channels (SICs) have been found amongst SACs. SICs have been identified in snail neurons, mammalian astrocytes, atrial myocytes and toad gastric smooth muscle. At low tensions when SACs are closed, SICs have been shown to be open. With increased tension SICs close and SACs open, indicating a mechanism of fine mechanosensitive control over K^+ at intermediate membrane tensions (Davies and Dull, 1993; Morris, 1990; Sackin, 1995). To our knowledge no attempt has been made to identify SICs in the eye.

8.2.4 The Cytoskeleton and Stretch-Activated Channels

SACs do not operate in isolation as their function is modulated by the cytoskeleton. In mammals the cytoskeleton couples to constituents of the plasma membrane such as channels, which include SACs. The cytoskeleton is also connected to the extracellular matrix and adjacent cells by specialized adhesions. The cytoskeleton imparts rigidity to cells and the plasma membrane, helping the cell withstand deforming forces and shear stress. Having this internal scaffolding means that eukaryotic cells do not need rigid walls and can more readily receive feedback from their physical surroundings.

The main cytoskeletal proteins are actin, intermediate filaments, and microtubules. Actin filaments, which determine cell shape and movement, are distributed throughout the cell, but especially just beneath the plasma membrane. Here it is tethered to membrane-bound ionic channels. Intermediate filaments are the cell's cable scaffolding, giving it mechanical strength to withstand shear stress and assist with motility. Microtubules direct intracellular traffic and determine where organelles lie. Integrins are membrane-spanning proteins at focal adhesions (for actin) or hemidesmosomes (for intermediate filaments) where cells contact the extracellular matrix. Integrin intracellular domains link to the cytoskeleton by many intervening proteins such as talin, alpha-actinin, and filamin. The cytoskeleton is further linked to the cytoskeleton of adjacent cells at cell–cell junctions via other intervening proteins such as cadherin. These linkages facilitate the forming of an interconnected structural unit, allowing concerted responses to mechanical stimuli.

The actin cytoskeleton attaches directly to the cell membrane, and its configuration and state of contractility can affect membrane-bound channel

activity and membrane potential (Sackin, 1995). Following the application of pressure or suction to a membrane patch the response lag noted in SAC activation and deactivation suggests that there may be an elastic component tunneling membrane tension towards the channels. This lag response is due to the viscoelastic actin network relaxing with time, transferring membrane tension to the SACs (Guharay and Sachs, 1984).

Agents that disrupt actin filament organization, such as the fungal toxin cytochalasin or colchicine, or agents that reduce cytoskeletal tension such as N-ethylmaleimide, have been shown to increase SAC activity (Patel et al., 1998; Sackin, 1995; Wan et al., 1999). In Lymnaea neurons, depolymerisation of the subcortical actin with cytochalasin-B, cytochalasin-D or treatment with N-ethylmaleimide enhances SAC activity (Small and Morris, 1994). In TRAAK or TREK-1-transfected cos-7 cells and tiger salamander retinal bipolar neurons, cytochalasin-D reduces activation delay time and enhances peak amplitude of voltage-gated K + channels (Maguire et al., 1998; Maingret et al., 1999; Patel et al., 1998); in chick skeletal muscle, cytochalasin-D increases stretch sensitivity 30-fold (Guharay and Sachs, 1984); in vascular endothelium cytochalasin reduces the delay time and shifts the open probability curve of SACs to lower pressures (Davies and Dull, 1993). Cochicine has been shown to enhance TRAAK channel activity and interestingly, intravitreal injection of this drug results in retinal atrophy (Davidson et al., 1983; Maingret et al., 1999). However, phalloidin which stabilizes actin, neutralizes the increased stretch sensitivity induced by cytochalasin (Maguire et al., 1998; Sachs, 1987). In excised patches of TRAAK-transfected cos-7 cells lacking a cytoskeleton connection the threshold for channel activation is markedly reduced and channel-activity enhanced (Maingret et al., 1999). These results strongly implicate the actin cytoskeleton in modulating SAC activity and mechanosensitivity.

8.3 Effects of Pressure on Eye Cells and Possible Relevance to the Pressure-Related Disease of Glaucoma

Glaucoma is an optic neuropathy that has as its basis slowly progressive loss of retinal ganglion cells and their axons. Elevated intraocular pressure is implicated in glaucomatous optic neuropathy. Intraocular pressure is determined by the equilibration of aqueous humour production by the ciliary body and aqueous outflow through the trabecular meshwork and uveoscleral pathways. Cells within the intraocular environment are continuously exposed to variations in intraocular pressure, the effects of which may be mediated by SAC and non-SAC mechanisms.

Apoptosis has been implicated in retinal ganglion cell death in glaucoma (Kerrigan et al., 1997; Okisaka et al., 1997; Quigley et al., 1995). Recent studies have localized TRAAK to the retina, cell bodies and axons of retinal ganglion

cells, dendrites of amacrine cells and outer segments of photoreceptors (Fink et al., 1998; Reyes et al., 2000). In vitro studies show that TRAAK, a mechanogated K^+ channel, is opened by membrane stretch, stimulated by arachidonic acid, naturally-occurring long-chained polyunsaturated free fatty acids and alkali conditions, and inhibited by gadolinium, amiloride and high concentrations of barium (Fink et al., 1996; Kim et al., 2001; Maingret et al., 1999, 2000; Maguire et al., 1998; Patel et al., 2001). Immunohistochemistry studies have shown TRAAK to be expressed in the immortalized retinal ganglion cell line, RGC-5 (Coroneo et al., 2002). TRAAK channels do not open at atmospheric pressure but require pressures in the order of –25 to –50 mmHg to induce half maximal channel opening (Maingret et al., 1999, 2000).[41,47] This is the pressure range that is clinically relevant to glaucoma and in vitro models of pressure-induced retinal ganglion cell loss. In vitro experiments undertaken in our laboratory show that arachidonic acid induces apoptosis in RGC-5 cells that can be attenuated by gadolinium or elevated extracellular potassium (unpublished data). Taken together these findings suggest a role for the SAC TRAAK in mediating RGC responses to pressure. Another SAC TREK-1 expresses higher levels of mRNA in glaucomatous optic nerve head astrocytes than in non-glaucomatous eyes. Similar stretch-activated K^+ channels have been described in bovine trabecular meshwork cells (Gasull et al., 2003), in which pressures required for channel opening are similar to those of RGC-5 cells. SACs have also been identified in human Muller glial cells (Puro, 1991).

It may be of interest that suction pressures required to open and activate SACs are in the range of pressures seen in clinical glaucoma and compression neuropathies (Lesage et al., 1996; Maingret et al., 1999; Morris, 1990; Patel et al., 1998). Normal carpal tunnel pressure is in the order of 10–13 mmHg, but in carpal tunnel syndrome it is in the order of 26–32 mmHg. Pressures in the order of 30 mmHg are reported in other entrapment neuropathies (Gelberman et al., 1981; Goodman et al., 2001; Luchetti et al., 1989; Szabo and Chidgey, 1989). SACs are also found on peripheral neurones and dorsal root ganglia (Bearzatto et al., 2000; Takahashi and Gotoh, 2000). Experimental studies report that compressive pressures of 30 mmHg or more inhibit fast axonal and retrograde transport under compression and induce morphological changes such as eccentric migration of the cell nucleus, decreased nuclear to cytoplasmic ratio and dispersion of Nissl substance (Dahlin et al., 1987). Pressures above 50 mmHg can threaten neuronal viability (Gelberman et al., 1981). It seems possible that SACs have a role in transducing these elevated pressures to ultimately influence cell behaviour.

There is in vitro evidence that elevated hydrostatic pressure directly affects retinal ganglion cells and their support astroglia (Tezel and Wax, 2000; Wax et al., 2000; Yang et al., 1993). Apoptosis is increased in cultured retinal ganglion cells and other types of central nervous system cells subjected to hydrostatic pressures mimicking conditions in acute (100 mmHg) and chronic (30 mmHg) glaucoma (Agar et al., 2000, 2001, 2006; Coroneo et al., 2001). In

these experiments, higher pressures were associated with more apoptosis. Cultured lamina cribrosa astroglia, when exposed to raised hydrostatic pressure, release tumour necrosis factor-alpha and nitric oxide, both pro-apoptotic substances. If retinal ganglion cells are co-incubated under normal pressure conditions with the previously pressurized glia, the retinal ganglion cells undergo apoptosis (Tezel and Wax, 2000).

Stretched trabecular meshwork cells in vitro activate high conductance Ca^{2+}-activated K^{+} channels (Gasull et al., 2003), an effect similar to that stimulated by volume changes in trabecular meshwork cells. The cells elongate and rearrange their actin filaments (Tumminia et al., 1998; Wax et al., 2000). There is upregulation of genes controlling inflammation, secretion, extracellular matrix digestion, oxidative stress responses, and cytoskeleton organization (Gonzalez et al., 2000). Levels of alpha-B-crystallin are reduced, which may contribute to stabilizing and regulating actin (Mitton et al., 1997). Signal transduction in the form of increased paxillin phosphorylation in focal adhesion complexes and MAP-kinase signaling is seen (Tumminia et al., 1998). Elevated hydrostatic pressure induces rounding of trabecular meshwork and pigmented and non-pigmented ciliary epithelium cells with associated changes in the actin cytoskeleton and adenylyl cyclase activity (Wax et al., 2000).

Mechanical stretching of cultured trabecular meshwork cells induces changes in the extracellular matrix as there is increased activity of matrix metalloproteinases (MMP) such as MMP-2, MMP-14, stromelysin and gelatinase A, and reduced levels of the tissue inhibitor of MMP (TIMP-2) (Bradley et al., 2001, 2003; WuDunn, 2001). The effects reverse when mechanical stretching is stopped. These pressure-induced alterations in MMP activity and extracellular matrix degradation may affect trabecular meshwork outflow resistance and have an important role in intraocular pressure regulation.

8.4 Conclusion

Ocular cells by virtue of their position within a mechanically active environment are subject to many physical forces. Examples of these forces include stretch, shear and pressure. SACs are vital components of the cell's mechanosensory apparatus. SACs do not function in isolation but are affected by perturbations and cellular events involving the plasma membrane, cytoskeleton, adhesion complexes and extracellular matrix. Cell–cell and cell-matrix interconnectivity through cytoskeleton-adhesion complexes means that responses to any mechanical stimulus need not be restricted to a single cell but can be orchestrated as cellular syncytia to the level of the whole tissue.

Various lines of evidence point to the putative role of SACs in sensing pressure and stretch in the eye. The capacity to be mechanosensitive likely confers upon ocular cells and tissues a degree of protection from injury and the ability to adapt to new environments. When these protective mechanisms

are exceeded then damage and disease ensue, such as in glaucoma. These basic mechanisms as they apply to the eye are only beginning to be understood. Furthering this understanding offers the prospect of fresh insights into disease pathogenesis and perhaps even a rational basis for new treatments.

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Chapter 9
Neuromodulation of Mechanosensory Input to the Insect CNS

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Abstract Mechanosensory input to the insect central nervous system (CNS) is mediated by cholinergic neurones. There is good evidence that the gain of such inputs can be modulated under different physiological conditions. This review focuses upon identification of the neuromodulators involved and the mechanisms by which they influence transmission mediated at cholinergic synapses.

Keywords Mechanosensory input · insect central nervous system · neuromodulators

9.1 Introduction

Neuromodulation is a fundamental feature of the nervous system. It allows animals to switch between different behavioral sets and to act appropriately under changing environmental conditions (for an overview of neuromodulation in invertebrates see reviews by Torkkeli and Panek, 2002; and Birmingham and Tauck, 2003). Although sensory receptors provide the nervous system with information that is essential for awareness of the world around and of the movements of different parts of the body, in many instances, the gain with which they operate and the impact of the signals they generate can be modulated. For example, we are all aware of changes in our attention, such that one or more sensory modalities may be filtered out, while we focus upon one particular sense. This kind of modulation is essential if the CNS is to obtain detailed information while not being swamped with information that is irrelevant in the current context. Neuromodulators are responsible for altering the excitable properties of neurones or the strength of synaptic connections and thereby reconfiguring the functional properties of neuronal networks (Katz and Frost, 1996). Upgrading or downgrading the priority of particular sensory lines can occur at different stages of sensory processing, starting with the

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receptors themselves and operating at multiple levels by a number of mechanisms within the CNS. It should also be remembered that sensory receptors are not merely reporters of the situation of the body and its environment; they can have a fundamental action in regulating motor activity. The role of sensory receptors in initiating reflex responses is well documented, but they may also have a profound influence on patterns of locomotor activity.

In this review modulation of mechanosensory input to the insect CNS is examined. The review focuses upon identification of the compounds involved in modulating cholinergic transmission at insect central synapses and the mechanisms underlying this neuromodulation. Particular consideration is given to neuromodulation in the flight motor system of the locust. The flight of the locust has come to be regarded as a classic example of a behaviour whose basic pattern is controlled by the central nervous system (Burrows 1975). The locust flight system thus represents a model for the investigation of neuromodulation. In this review we focus on neuromodulation of mechanosensory information from the wing stretch receptors (SR), which have been studied extensively because of their major role in insect flight. However, in addition to their importance in the context of flight, they illustrate a number of basic features, which may be widespread among different types of sensory receptor. Studies on the 'fast' coxal depressor motoneurone of cockroach (D_f) are also reviewed since this system has been used to establish the detailed cellular mechanisms underlying modulation of cholingergic synaptic transmission. Because acetylcholine is the principal sensory neurotransmitter in insects, such modulation will have fundamental implications to the handling of sensory information and the impact it has on motor output. The work presented illustrates that sensory input to the CNS can be influenced at several levels and by a range of mechanisms, including changes in the responsiveness of the sensory receptors, changes in the efficiency of the synapses afferent nerves make with central neurones or alterations in the excitability of those central neurones. Although there is evidence that the response of SRs to wing movement can be modulated, the major focus will be upon changes within the CNS.

9.2 Presynaptic Modulation of Mechanosensory Input

Presynaptic modulation of sensory information is a universal phenomenon that serves to alter the effectiveness of signalling between presynaptic and postsynaptic neurones by changing the amount of neurotransmitter released from the presynaptic afferent terminals onto the postsynaptic cell. It is potentially a very selective form of modulation as it provides a mechanism of controlling the inflow of sensory information from the same or different modalities without altering the responsiveness of the postsynaptic cell to other inputs. Presynaptic inhibition of sensory input is one of the most widely studied mechanisms of modulating sensory information in mechanosensory afferents in insects.

9.2.1 Transmitters Involved in Presynaptic Inhibition

Ultrastructural and electrophysiological evidence indicate that γ-aminobutyric acid (GABA) is the predominant neurotransmitter involved in presynaptic inhibition at insect sensory afferents (see Watson, 1992; and Burrows, 1996). It has been identified by immunocytochemistry in processes presynaptic to mechanosensory afferents in locusts (Watson and Pflüger, 1984; Watson, 1990; Watson and England, 1991; Watson et al., 1991, 1993; Judge and Leitch, 1999a; Richardson and Leitch, 2007); and also other insects including cockroaches, bees, crickets and moths (for review see Staudacher et al., 2005).

In locusts, immunoreactivity for GABA or its synthetic enzyme glutamate decarboxylase has been demonstrated in processes presynaptic to the terminals of prosternal filiform afferents (Watson and Pflüger, 1984); cercal afferents (Watson, 1990); campaniform sensilla (Watson and England, 1991); hair plates (Watson et al., 1991); femoral chordotonal organ afferents, (Watson et al., 1993); and wing stretch receptors (Judge and Leitch, 1999a; Richardson and Leitch, 2007). Furthermore, electrophysiological studies have provided functional evidence for GABA mediated presynaptic inhibition at these sensory terminals (locust filiform hair afferents, Boyan, 1988; locust femoral chordotonal organ afferents, Burrows and Laurent, 1993; and the locust wing stretch receptors Judge and Leitch, 1999b).

However, other neurotransmitters have also been identified in processes presynaptic to locust mechanosensory afferents. Of the different classes of identified input to prosternal hair afferents, 51% have been reported to be immunoreactive for GABA, 40% for glutamate, and 9% for other unidentified neurotransmitters (Watson and Pflüger, 1984). Immunocytochemical identification of neurotransmitters involved in modulation of transmitter release from the central terminals of wing stretch receptors have revealed that 55% of all inputs to the forewing stretch receptor (fSR) are immunoreactive for glutamate (Richardson and Leitch, 2007). Clearly GABA and glutamate are the major neurotransmitters involved in presynaptic modulation of transmitter release from locust mechanosensory afferents. Both GABA and glutamate are widely distributed throughout the locust CNS (Bicker et al., 1988, Watson, 1988) and have been shown to have inhibitory effects on flight neurones (Dubas, 1990, 1991). Nevertheless, there is evidence that other transmitters such as acetylcholine and biogenic amines are involved in neuromodulation at mechanosensory afferent synapses. Furthermore, experiments by Dubas (1991), in which the effects of pressure applications of glutamate, GABA, aspartate, taurine, glycine and cysteine were studied using a locust preparation in which neuropile intracellular recordings could be made during expression of the flight motor output, indicated that the majority of cells responded to glutamate, GABA, aspartate and taurine. These experiments indicated that receptors for these putative amino acid neurotransmitters are present on the dendritic arborizations of flight motoneurones.

Acetylcholine, which is found in very high levels within insect nervous tissue, appears to be the major neurotransmitter used by insect sensory neurones (Pitman, 1985) to excite postsynaptic neurones (Lutz and Tyrer, 1988; Tyrer et al., 1984) and has been shown to act mainly on nicotinic receptors. Nicotinic acetylcholine receptors are present throughout the locust CNS (Leitch et al., 1993). However, acetylcholine has also been reported to mediate presynaptic inhibition of mechanosensory afferents themselves (Trimmer and Weeks, 1989). Results from larval *Manduca* (Trimmer and Weeks, 1989) suggest that there is a negative feedback loop from the postsynaptic neurone to the afferent via muscarinic autoreceptors located in the axon terminals of the afferents. A functional role for muscarinic receptors in down-regulating the release of acetylcholine from mechanoreceptors in locusts is discussed further under "*Mechanisms of Presynaptic Inhibition*".

The biogenic amines octopamine, serotonin, and dopamine have also been shown to be important in the modulation of insect flight (Claassen and Kammer, 1986; Whim and Evans, 1988; Ramirez and Orchard, 1990; Orchard et al., 1993; Stevenson and Meuser, 1997; Duch and Pflüger, 1999), and thus are potential candidates as neuromodulators at locust mechanosensory synapses. However direct anatomical evidence demonstrating biogenic amines in processes presynaptic to mechanosensory afferent terminals in insects has been more difficult to achieve. Recently, Richardson and Leitch (2007) were able to identify octopaminergic neurones in the locust central nervous system containing distinctive large, electron-dense granules (Fig. 9.1), which could reliably be used to identify them. Although putative octopaminergic processes were found in close contact to labelled stretch receptor profiles, no morphologically recognizable synaptic inputs to the stretch receptor terminals were evident. They concluded that aminergic neurones, that modulate the synapse, may have very few morphologically recognizable synaptic outputs. Despite the lack of direct anatomical evidence of aminergic synaptic inputs in the insect CNS there is clear electrophysiological evidence for octopaminergic modulation of synaptic transmission between sensory afferents and flight motoneurones, such as the first basalar motoneurone (BA1), in the locust (Leitch et al., 2003). This amine has been shown to modulate cholinergic responses in the cockroach 'fast;' depressor motoneurone via a mechanism involving intracellular second messenger signalling (Butt and Pitman, 2002). The mechanisms underlying the action of these biogenic amines in the modulation of mechanosensory information is discussed under the later section on "*Postsynaptic Modulation of Mechanosensory Input* ".

Potential neuronal sources of octopamine in the locust CNS are the octopaminergic dorsal unpaired median (DUM) neurones (Watson, 1984). Octopamine is released from DUM neurones during flight, walking, and jumping (Ramirez and Orchard, 1990; Orchard et al., 1993; Duch and Pflüger, 1999). The DUM neurones project into the same dorsolateral neuropile region of the CNS into which the fSR and the flight motoneurone BA1 project. Octopamine immunoreactivity is also present in this region, so the DUM neurones are

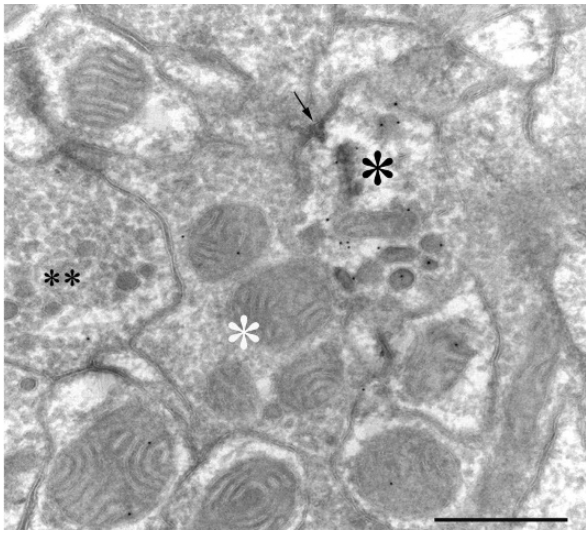

Fig. 9.1 Electron micrograph of a transverse section from a mesothoracic ganglion labeled with an anti-octopamine antibody and prepared by a cryoimmunogold labeling technique. Three different profiles can be distinguished on the basis of the vesicles they contain. One contains small clear vesicles (white asterisk); another contains spherical dense-cored vesicles (small black asterisks). Only the profile with the cigar-shaped vesicles (large black asterisk) is labeled with gold particles. Note (black arrow) that one of the spherical dense-cored vesicles appears to be fusing with the plasma membrane; this could be an indication of nonsynaptic exocytosis. Scale bar _ 500 nm. Taken from Fig. 9.8. Richardson and Leitch (2007). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

strong candidates as the source of the octopamine that may potentially modulate transmission across the fSR/BA1 synapse. A group of octopaminergic DUM neurones, which are present in the subesophageal ganglion (Bräunig, 1991), have been identified by Bräunig and Burrows (2004) as a potential source for the modulatory effects of octopamine on central synapses of sensory neuronal projections. Bräunig and Burrows (2004) have suggested that the projection patterns of these putatively octopaminergic neurones is such that they could be the source of the octopaminergic modulation of networks underlying sensory processing and motor pattern generation within the locust thoracic ganglia.

9.2.2 Mechanisms of Presynaptic Inhibition

As previously stated, the flight motor system of the locust represents a model invertebrate preparation for the investigation of neuromodulation. At the hinges of the wings are stretch receptors, which are important elements in

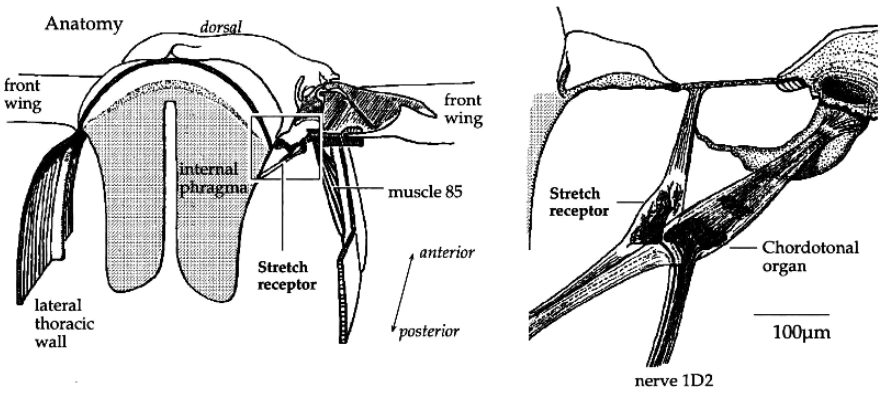

Fig. 9.2 The stretch receptor at a wing hinge showing the morphology of the thoracic skeleton and the position of the stretch receptor. The boxed area is magnified on the right to show the insertion of the single stretch receptor sensory neuron and the many sensory neurons of the nearby chordotonal organ. Taken from Fig. 11.14. Burrows (1996). Copyright still pending

generating and controlling the flight motor pattern (Fig. 9.2.). The single stretch receptor associated with each wing hinge (Gettrup, 1962) is a tonic proprioceptor signalling wing position; it fires phasically at the top of the wing stroke (Wilson and Gettrup, 1963). Each stretch receptor comprises a single cell body with associated dendritic arborizations located near the point of articulation of the wing with the thorax (Altman and Tyrer, 1977). As the wing is raised, the stretch receptor dendrites are stretched, thus initiating spikes that are conducted towards the central nervous system. Alterations in wing position are signalled by changes in the number of spikes, instantaneous spike frequency, spike burst duration, and its phase during the wing beat cycle. Each wing stretch receptor projects a single axon into the CNS (Gray and Robertson, 1996) where it branches extensively (Fig. 9.3) and makes monosynaptic connections to depressor motoneurones innervating that wing and also with flight interneurones (Burrows, 1975). Mechanosensory information about the angle of each individual wing is thus provided entirely by a single mechanosensory neurone rather than by a population of such neurones. Modulation of neurotransmitter release from the terminals of SR neurones has been examined at identified synapses between forewing stretch receptors and the first basalar motoneurone BA1 in the locust *Schistocerca gregaria* (Leitch and Pitman 1995; Judge and Leitch 1999a,b; Leitch et al., 2003; Richardson and Leitch, 2007). Studies by Leitch and Pitman (1995) and Judge and Leitch (1999b) demonstrated that the synapse between the stretch receptor and the flight motoneurone BA1 is cholinergic and that release of acetylcholine from the presynaptic terminals of the stretch receptor is down-regulated by activation of presynaptic muscarinic receptors.

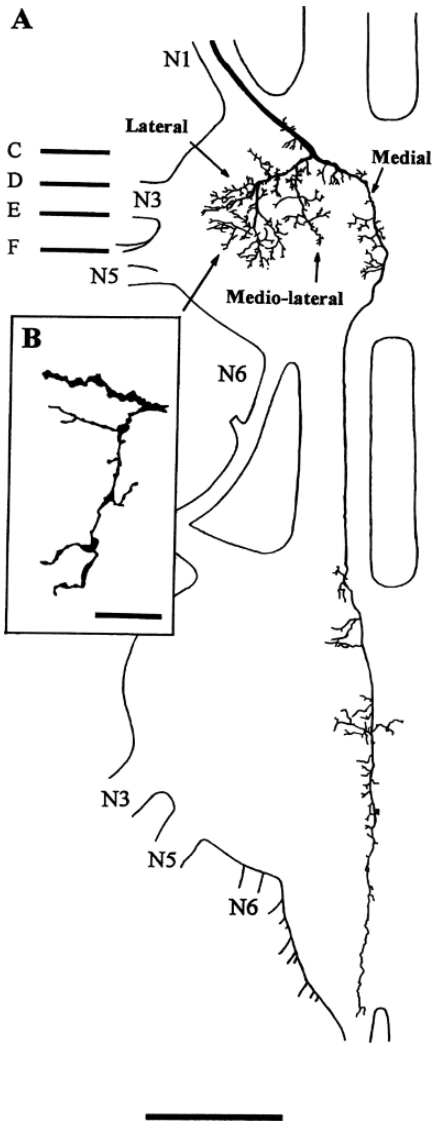

Fig. 9.3 **A**: Camera lucida drawings of the locust forewing stretch receptor (fSR) taken from a wholemount preparation showing the position of the fSR within the mesothoracic and metathoracic ganglia. Anterior is to the top. After entering the mesothoracic ganglion via nerve 1 (N1), the fSR divides into a medial branch and a lateral branch. The medial branch runs close to the dorsal midline, into a meso-metathoracic connective, and then into the metathoracic ganglion. The lateral branch bifurcates into a medio-lateral and lateral branch. The medio-lateral branch terminates before the emergence of nerve 4 (N4) and nerve 5 (N5). The extensive lateral branch aborizations extend posteriorly to the emergence of N4 and N5. **B**: High magnification of the fine terminal branch indicated by the arrow in A. Note the characteristic swellings along its length. Scale bars = 200 μm in A, 20 μm in B. Taken from Fig. 9.1. Judge and Leitch (1999). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

A functional role for muscarinic receptors in down-regulating the release of acetylcholine was first shown in a locust (*Locusta migratoria*) synaptosome preparation (Breer and Knipper, 1984; Knipper and Breer, 1988). Direct evidence that muscarinic receptors play an important role in modulating synaptic transmission at insect synapses was provided by in electrophysiological studies in a variety of insect preparations including: the cockroach cercal afferent-giant interneurone synapses (Hue et al., 1989; Le Corronc et al., 1991; Le Corronc

and Hue, 1993); the tobacco hornworm planta hair afferent proleg motoneurone synapses (Trimmer and Weeks, 1989, 1993); the locust forewing stretch receptor neurone and first basalar motoneurone synapses (Leitch et al., 1993; Leitch and Pitman, 1995); and the locust campaniform sensilla afferent-fast extensor tibiae motoneurone synapses (Parker and Newland, 1995). The presynaptic muscarinic receptors involved in the depression of acetylcholine release from insect sensory afferent terminals were assumed to be located on the afferent terminals themselves where they acted as autoreceptors. However, Leitch and Pitman (1995) and Judge and Leitch (1999b) suggested an alternative/additional possibility that presynaptic muscarinic receptors could be located on inhibitory interneurones that receive inputs from collaterals of the sensory afferent. They hypothesised that acetylcholine released by stimulation of the sensory neurone could activate the muscarinic receptors on the interneurones, which in turn would be stimulated to release inhibitory neurotransmitter into the sensory afferent terminal and thereby reduce further release of acetylcholine. Judge and Leitch (1999b) were able to demonstrate that cholinergic muscarinic receptors on presynaptic GABAergic neurones are involved in downregulating ACh release from the fSR terminals. Using electrophysiological and pharmacological techniques they were able to show that electrical stimulation of the fSR evokes monosynaptic excitatory postsynaptic potentials (EPSPs) in the flight motoneurone BA1 mainly by the activation of postsynaptic nicotinic cholinergic receptors. Application of the general muscarinic antagonists scopolamine and atropine caused a reversible increase in the amplitude of electrically evoked EPSPs. When the GABA antagonist picrotoxin, which has been successfully used to block the effects of applied or synaptically released GABA on other insect neurones (Burrows and Laurent, 1993; Lees et al., 1987; Pitman and Kerkut, 1970; Sattelle et al., 1988; Watson and Burrows, 1987) was applied to the fSR/BA1 preparation it also caused a reversible increase in the EPSP amplitude, that was not due to an increase in sensitivity of BA1 to ACh, as picrotoxin did not increase ACh responses recorded from BA1. When scopolamine was applied to a preparation that had been preincubated with picrotoxin, the EPSP amplitude enhancement normally seen in control experiments did not occur; in fact, it caused a slight depression (Fig. 9.4). These experiments indicated that at least some of the presynaptic muscarinic receptors were located on GABAergic interneurones that modulate transmission at the fSR/BA1 synapse. Electron microscopical immunocytochemistry studies confirmed that GABA-immunoreactive neurones make presynaptic inputs to the fSR (Judge and Leitch, 1999a). Results from studies on the cockroach cercal afferent-giant interneurone preparation also lends support to the possibility that at least some of the presynaptic muscarinic receptors identified in other insect preparations may also be located on GABAergic interneurones. Workers have shown that GABAergic interneurones involved in the modulation of ACh release from the cercal afferent terminals are partly activated by muscarinic agonists (Le Corronc et al., 1991).

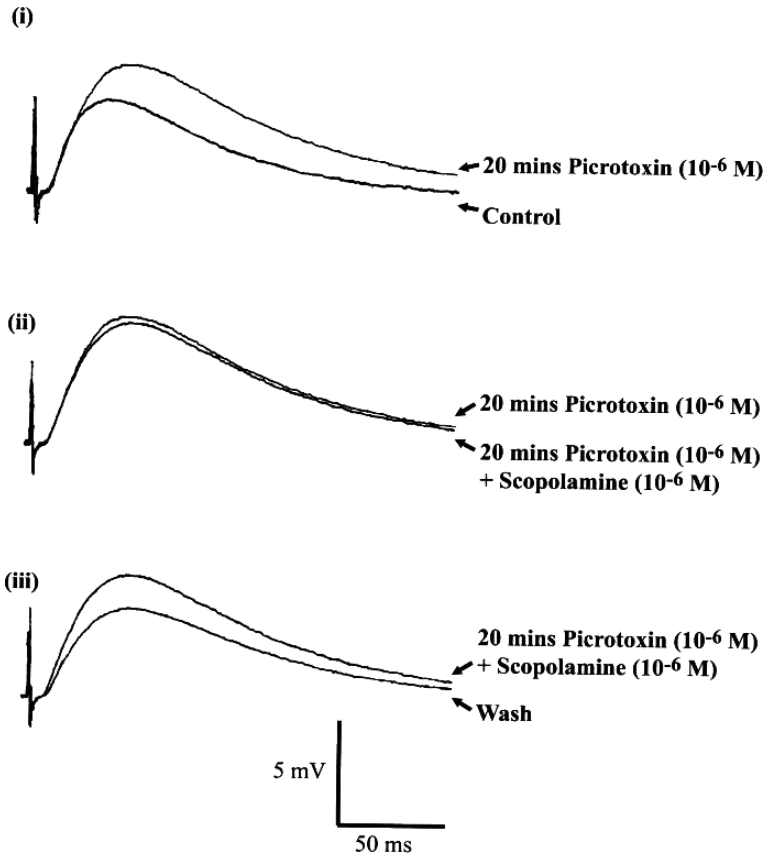

Fig. 9.4 The effect of the general muscarinic antagonist, scopolamine (10^{-6} M) on electrically evoked EPSPs recorded from BA1 in a preparation preincubated with the GABA antagonist picrotoxin (10^{-6} M). (**i**) Preincubating the preparation with picrotoxin (10^{-6} M) resulted in a marked increase in the amplitude of electrically evoked EPSPs. (**ii**) Applying scopolamine (10^{-6} M) to the same preparation caused a slight reduction in EPSP amplitude. It did not cause an increase in the EPSP amplitude as seen in control experiments in the absence of picrotoxin. (**iii**) Perfusing the preparation with fresh saline caused the EPSP amplitude to return to control levels. Taken from Fig. 9.5. Judge and Leitch (1999). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

9.2.3 Distribution and Abundance of Inhibitory Synapses on Afferent Terminals

Studies into the distribution and abundance of different types of neuromodulatory inputs to mechanosensory afferents in insects have been accomplished by first backfilling the peripheral sensory structure with electron-dense dyes (e.g. cobalt salts, horseradish peroxidase (HRP) or neurobiotin) to reveal the central

terminal arborizations and then immunolabelling with antibodies against different neurotransmitters followed by immunogold.

Analysis of the proportion of processes presynaptic to the fSR which are immunoreactive (IR) for GABA has revealed that 43% of all presynaptic inputs to the fSR central terminals are GABAergic (Judge and Leitch, 1999a). The majority (54%) of identified GABAergic inputs are located on the lateral field of branches of the fSR (Fig. 9.5). However, there is no evidence that different types of synaptic input are exclusively located in any particular region of the arborisation; GABA-IR and non-immunoreactive inputs are intermingled on all branches. GABAergic inputs are also intermingled with output synapses on terminal branches of the stretch receptor afferents, frequently in close proximity to each other, suggesting that presynaptic control of transmitter release is

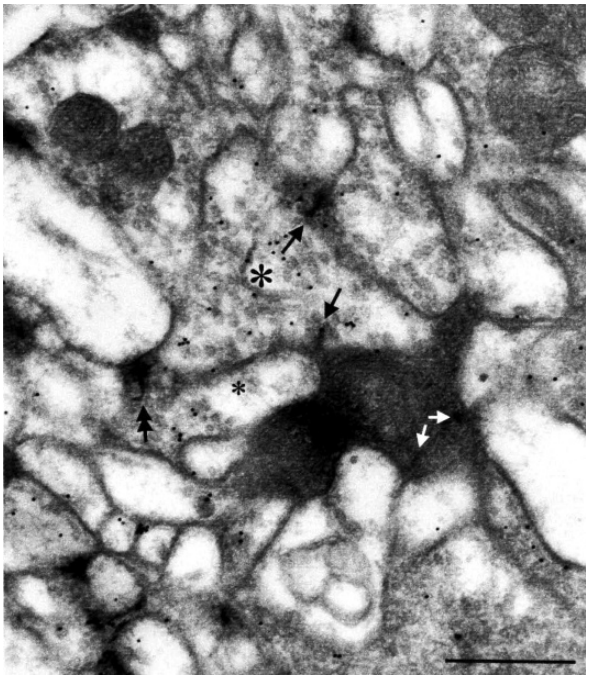

Fig. 9.5 Electron micrographs of transverse sections through the mesothoracic ganglion showing profiles of the lateral forewing stretch receptor (fSR) branch labelled with horseradish peroxidase. The electron-dense fSR profiles receive inputs from GABAimmunoreactive (GABA-IR) processes, indicated by a high density of gold beads. The GABA-IR process (large asterisk) is making input synapses (single black arrowheads) onto a fSR process and other GABA-IR processes. One of the postsynaptic GABA-IR processes (small asterisk) makes a reciprocal synapse (double arrowhead) back onto the former GABA-IR process (large asterisk). The fSR process makes output synapses (white arrowheads) onto nonimmunoreactive profiles. Scale bar = 0.5 μm. Taken from Fig. 9.2B. Judge and Leitch (1999). Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

precisely targeted. A similar arrangement of input and output synapses has been reported in other types of insect sensory afferents e.g. locust hindwing receptor (Altman et al., 1980); prosternal filiform hairs (Watson and Pflüger, 1984); campaniform sensilla (Watson and England, 1991); hair plates (Watson et al., 1991); and femoral chordotonal organ (Watson et al., 1993). However, the proportion of inputs that are GABA-IR varies considerably in different insect preparations. Many of the mechanosensory afferents that have been analysed in locusts have a markedly higher proportion of GABA-IR inputs than the 43% reported at fSR terminals. For example: 93% of inputs to hair plate afferents (Watson et al., 1991); 72% of inputs to campaniform sensilla afferents (Watson and England, 1991); 78% of inputs to femoral chordotonal organ afferents (Watson et al., 1993); and 51% of inputs to prosternal hair afferents have been reported to be immunoreactive for GABA. The significance of the differences in the proportion of GABA-IR input to each of these different types of sensory afferent is unclear at present but may indicate that presynaptic inhibition plays a relatively more important role in modulation of some afferent outputs. Locust hair plates and campaniform sensillae, each comprise only a few tens of sensory neurones and for many of the reflex pathways in which they are involved, sensory information is not processed by a large parallel array of interneurones but rather passes directly to individual motoneurones with unique roles (Pearson et al., 1976) or to a limited number of premotor non-spiking interneurones (Burrows and Pflüger, 1988). Thus in the case of these afferents, presynaptic inhibition alone, would be an appropriate mechanism of sensory gating (Watson and England, 1991). In the locust fSR a higher proportion (57%) of inputs are not-immunoreactive for GABA indicating that the effectiveness of synaptic transmission from the fSR afferent could also be modulated by inputs from other non-GABAergic neurones.

9.3 Postsynaptic Modulation of Mechanosensory Input

9.3.1 Alterations in Neurone Membrane Properties

The classical view of the way in which one neurone influences another is that neurotransmitter causes opening of particular ion channels that will increase membrane conductance. Whether the neurotransmitter has an excitatory or inhibitory action will depend upon the position of reversal potential of the ions involved relative to the threshold of the neurone. If the neurotransmitter is excitatory, essentially linear summation of postsynaptic potentials is required for action potential threshold to be reached. Inhibitory neurotransmitters will reduce the effectiveness with which such summation will attain threshold mainly by increasing membrane conductance, so short-circuiting the effects of any excitatory input. It is now clear that this view has had to be modified to take into account other changes that can be caused by neurotransmitters and

neuromodulators. For example, neurotransmitters can modulate the actual properties of neurones such that the effects of inputs can be changed not merely quantitatively but also qualitatively. Thus the electrical properties of some locust interneurones and motoneurones can be significantly changed under certain circumstances. For example octopamine can induce some interneurones and motoneurones to produce plateau potentials (Ramirez and Pearson, 1991a,b). Such plateau potentials can increase the tendency of a neurone to generate action potentials in bursts rather in a more distributed manner. The effect of this will normally be to enhance rhythmicity in a network and its output. Similar plateau potentials have also been recorded from the 'fast' coxal depressor motoneurones in an isolated cockroach nerve cord preparation, although in this preparation they can be observed in the absence of applied amines (Hancox and Pitman, 1991). (Figure 9.6) Since the electrical properties

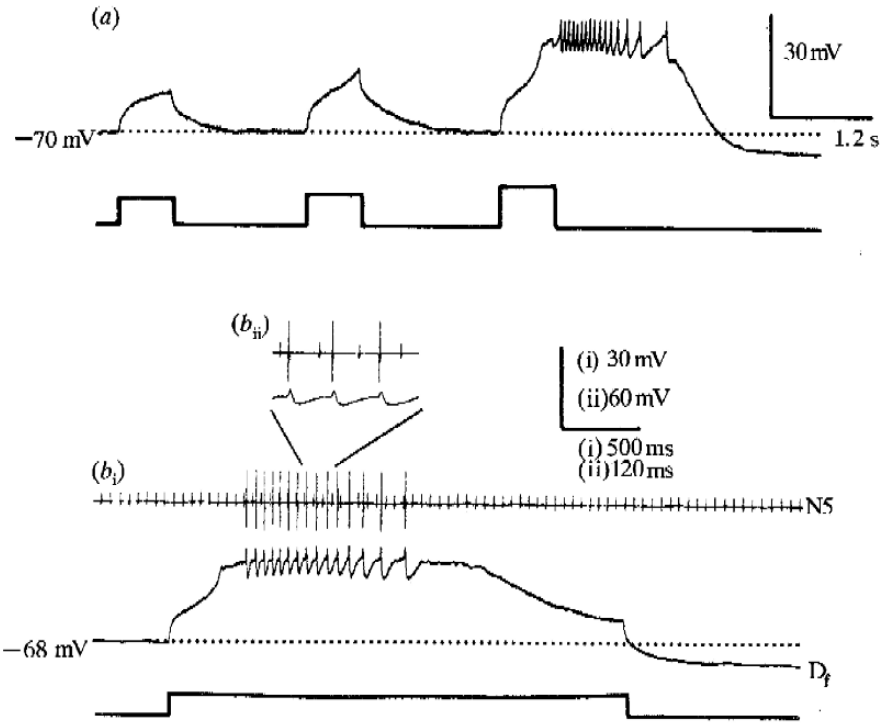

Fig. 9.6 Plateau potentials recorded from the cockroach 'fast' coxal depressor motoneurone (D_f). (**a**) Responses to 1 nA, 1.2 nA and 1.5 nA depolarizing pulses applied to the neurone soma, demonstrating that the plateau potentials have a distinct threshold. Dotted line indicates resting potential. (**bi**) Simultaneous recordings made intracellularly from the soma of D_f (middle trace) and extracellularly from nerve 5 (containing the axon of D_f) (upper trace) showing that action potentials driven by a plateau potential propagate along the axon of D_f. (**bii**) Display on an expanded scale shows that axonal action potentials slightly precede attenuated action potentials recorded from the neurone soma. Taken from Fig 9.1. Hancox and Pitman (1991)

of motoneurones in this preparation change over time (Hancox and Pitman, 1992), it is possible that these plateau potentials were enabled by some change (e.g. stress-induced hormone release) associated with setting up the preparation for recording.

At least some insect neurones, including the first basalar motoneurone of the locust (Anderson and Pitman, 1994) and the fast coxal depressor motoneurone (D_f) of the cockroach third thoracic ganglion (David and Pitman, 1993) possess at least two classes of ACh receptor. One of these has many characteristics in common with classical nicotinic receptors in other preparations such as those of vertebrate skeletal muscle and the electric organ of *Torpedo* (David and Sattelle, 1984, 1990). In the cockroach D_f motoneurone, the second type of ACh receptor has a pharmacology that does not allow it to fit neatly into the vertebrate classification scheme; it is activated by a range of muscarinic agonists, but is also sensitive to nicotine. On the other hand, this receptor is blocked by a range of vertebrate muscarinic receptor antagonists but not by the nicotinic antagonist α-bungarotoxin (David and Pitman, 1993). Despite the agonistic action of nicotine on this type of receptor, in light of their wider pharmacological profile and evidence that they exert their effects via second messengers David and Pitman (1994, 1996c) classified them as muscarinic ACh receptors (for an explanation of the terminology of these receptors see David and Pitman, 1996a). Early studies on insect neurones focussed largely on the nicotinic ACh receptors, because they generate marked membrane depolarization and excitation when cholinergic agonists are applied at the normal resting potential of a neurone (Kerkut et al., 1969; David and Sattelle, 1984, 1990). The effects of insect muscarinic receptors, on the other hand, were far more elusive; unlike nicotinic agonists, muscarinic agonists have little effect when applied to neurones. It turns out that muscarinic receptors have more subtle actions, among which include the modulation of voltage-gated channels. In the cockroach D_f motoneurone activation of muscarinic receptors causes suppression of calcium currents (David and Pitman, 1996a), which in turn causes a reduction in the amplitude of calcium-dependent K currents (I_{KCa}) which are probably the largest current component evoked in this neurone by depolarization (Thomas, 1984; David and Pitman, 1995). It is likely, therefore, that muscarinic receptors may mediate changes in the electrical properties, and hence the excitability and overall responsiveness of this neurone to synaptic input.

9.3.2 Modulation of Ligand-Gated Ion Channels

There is evidence that certain synapses can exert more selective actions than those seen at classical synapses. In such cases the overall excitability of a particular neurone may remain unaltered, while the effectiveness of particular inputs may be selectively enhanced or depressed. This type of alteration is illustrated by the interaction between muscarinic and nicotinic effects of ACh

in individual neurones and by the effects of monoamines on transmission mediated by nicotinic ACh receptors; muscarinic receptors can mediate alterations in the efficacy of ligand-gated ion channels. Application of the muscarinic agonist McNeil-A-343 (McN) selectively activates muscarinic receptors in the cockroach D_f motoneurone without stimulating nicotinic receptors. However, application of McN suppresses nicotinic ACh responses (David and Pitman, 1996b) (Fig. 9.7). This action of McN is prevented by administration of the muscarinic receptor antagonist pirenzepine, which, when applied alone has no effect on ACh responses mediated through nicotinic receptors. Since these experiments were performed under voltage-clamp conditions, in which ionic currents induced by nicotinic receptor activation were measured directly, any change in amplitude of these nicotinic responses could not be attributed to an indirect effect caused by a change in membrane conductance. Instead, the observations indicate that McN must change the number of nicotinic ion channels opened by a standardized amount of ACh.

In addition to modulating responses mediated by nicotinic ACh receptors, McN also modulates responses to GABA. In this preparation GABA acts upon ligand-gated ion channels with a pharmacological profile intermediate between those of vertebrate $GABA_A$ and $GABA_C$ receptors (Anthony, et al., 1993; Pinnock, et al., 1988). Vertebrate, nicotinic ACh ion channels and $GABA_A$ channels share numerous structural similarities, although each has its own distinct profile of ligand specificities. GABA responses could also be modulated by ACh applied in the presence of the nicotinic antagonist α-bungarotoxin, supporting the view that this modulation is indeed mediated by muscarinic receptors and is not dependent on functional nicotinic ACh receptors. For reasons which are, as yet, unclear, in some preparations, McN causes an

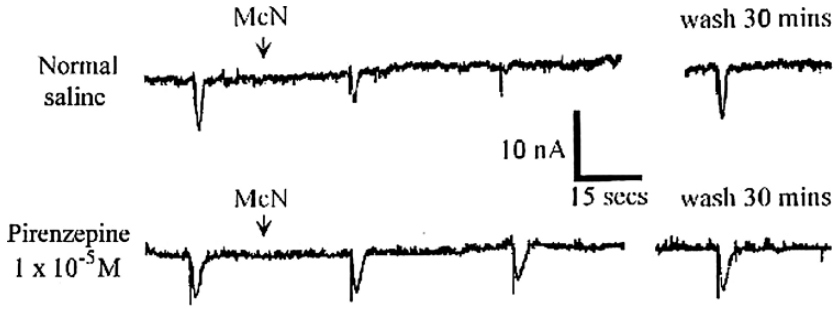

Fig. 9.7 Suppression of nicotinic inward currents evoked in the cockroach 'fast' coxal depressor motoneurone (D_f) by activation of muscarinic receptors. Nicotinic responses were evoked by regular brief pulses of ACh applied locally from a micropipette by pressure. (Holding potential = –80 mV). The top trace shows that bath application of the muscarinic agonist McNeil-A-343 (McN) (10^{-4} M) almost completely blocks the response to ACh. The bottom trace shows that effect of McN is specifically mediated by muscarinic receptors, since suppression of ACh responses is blocked by application of the muscarinic receptor antagonist pirenzepine (10^{-5} M). Taken from Fig. 9.1. David and Pitman (1996b)

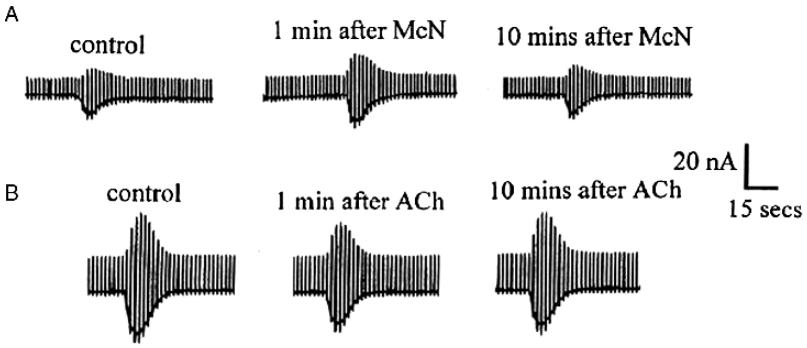

Fig. 9.8 Muscarinic receptor-mediated modulation of GABA-induced currents recorded from the cockroach 'fast' coxal depressor motoneurone (D_f). In these recordings the membrane potential was held at –110 mV and stepped to –70 mV at 11.5 s intervals. This allowed E_{GABA} to be constantly monitored. In some D_f neurones, activation of muscarinic receptors increases the magnitude of GABA currents (trace A), while in others this causes them to be attenuated (trace B). In trace (**A**) the McNeil-A-343 (McN) was used to stimulate muscarinic receptors, while in trace (**B**) ACh was applied in the presence of α-bungarotoxin, which blocks the nicotinic but not the muscarinic effects of ACh. Taken from Fig. 9.2. David and Pitman (1996b)

enhancement in the magnitude of GABA responses, while in other preparations the GABA response is reduced (Fig. 9.8). By repeatedly stepping the membrane potential of the neurone between two different holding potentials during the course of single GABA responses it was possible to eliminate the possibility that activation of muscarinic receptors merely altered the reversal potential of GABA responses (presumably by altering ionic gradients across the neurone surface membrane). As with muscarinic receptor-mediated modulation of nicotinic responses, modulation of GABA currents was observed under voltage-clamp conditions, eliminating the possibility that this effect was mediated indirectly, rather than by way of a change in activation of GABA-activated channels.

It had been shown previously that activation of muscarinic receptors in the cockroach ventral nerve cord causes a rise in turnover of inositol phosphates (David and Pitman, 1994). Since inositol trisphosphate (IP_3) normally exerts its actions via a rise in intracellular calcium ion concentration ($[Ca^{2+}]_i$), the effect of McN on the concentration of this ion were investigated. $[Ca^{2+}]_i$ changes were determined by monitoring changes in fluorescence of intracellularly injected of the calcium indicator Fluo-3. Under these conditions, application of McN caused a rapid rise in $[Ca^{2+}]_i$. After wash-out of McN, $[Ca^{2+}]_i$ returned to its resting level with a time course approximately ten times more rapid than the period over which McN had its modulatory effects on nicotinic or GABA currents (David & Pitman, 1996b). The crucial role of $[Ca^{2+}]_i$ in modulating nicotinic ACh and GABA currents was demonstrated by intracellular release of calcium ions by flash photolysis of injected nitr-5; this transient

rise in $[Ca^{2+}]_i$ mimicked the effects of applied McN (David and Pitman, 1996b). Further support for involvement of IP_3 in muscarinic modulation of nicotinic and GABA currents has also been provided by the effects of lithium ions (Li^+). These ions have been shown to prevent degradation of IP_3 in several insect preparations (Drummond and Raeburn, 1984; Trimmer and Berridge, 1985), so leading to a significant and sustained rise in intracellular $[IP_3]$. In the presence of Li^+ muscarinic receptor-mediated modulation of nicotinic or GABA responses (which normally reversed within 10–15 minutes of washing out McN from the preparation) became effectively irreversible over the time course of recordings (David and Pitman, 1996b). This observation would be expected if muscarinic receptors mediate their modulatory actions by way of a rise in $[IP_3]$, which, in turn, triggers a rise in $[Ca^{2+}]_i$. The fact brief intracellular pulses of calcium generate modulation of nicotinic ACh or GABA currents over a comparatively long time-course suggests that calcium does not act directly upon the ligand-gated ion channels themselves, but, instead, triggers a further chain of events. The most likely of these is that calcium activates a protein kinase C (PKC) that, in turn phosphorylates cellular elements, possibly the nicotinic and GABA gated ion channels themselves.

The monoamines 5-HT, dopamine an octopamine can be detected in the insect CNS by biochemical and histochemical methods (Evans, 1980; Pitman, 1985; Richardson and Leitch, 2007). They are present in a comparatively small number of neurones, which tend to have extensive branching in neuropils of the ventral nerve cord. It is clear that individual octopaminergic neurone can exert actions that may be important in specific behaviours (Orchard, et al., 1993; Libersat and Pflüger, 2004) although its exact cellular mode of action is not clear. Even less is known of the roles of dopaminergic and serotonergic neurones or of the mechanisms by which they may exert any physiological effects. It is clear, however, that, when applied exogenously to the 'fast' coxal depressor motoneurone (D_f) of the cockroach third thoracic ganglion, all three amines can produce significant modulation of nicotinic ACh responses. As with modulation induced by activation of muscarinic receptors, the amines reduced the magnitude of nicotinic responses without causing a significant change in membrane potential or membrane conductance (Fig. 9.9). Although octopamine, dopamine and 5-HT all have a similar modulatory effect, the mechanism of action of 5-HT has been studied most extensively. In this a pharmacological approach was employed because it eliminated problems associated with changes in the amount of neurotransmitter released from presynaptic terminals. The target for the suppressing action of amines on ACh responses is a class of nicotinic ACh receptor rather than a muscarinic receptor, since responses to locally applied nicotine are also suppressed by these amines. Because it was already known that muscarinic receptors can mediate suppression of nicotinic ACh responses, it was important to eliminate the possibility that amines were exerting their effects indirectly by way of muscarinic receptors. This possibility was eliminated, since amine-mediated suppression of ACh responses still occurred after muscarinic receptors had been pharmacologically blocked

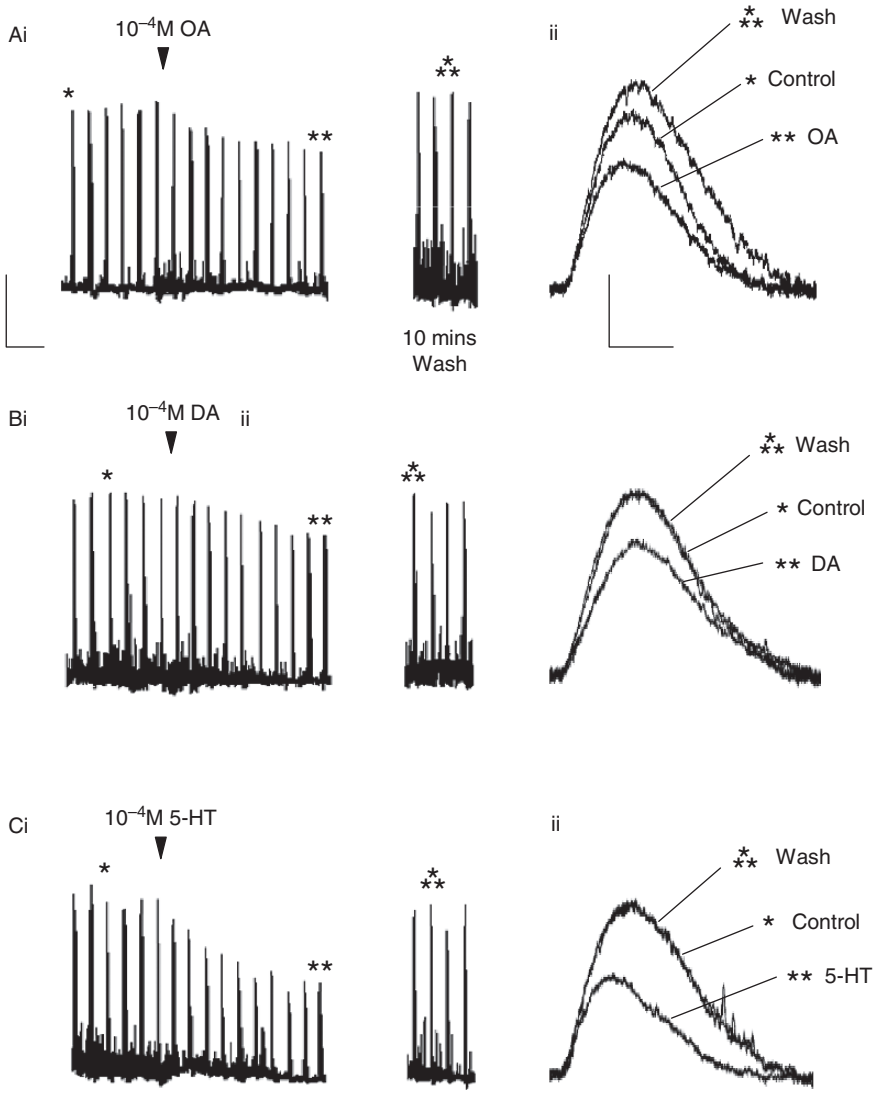

Fig. 9.9 Modulation by (**A**) octopamine, (**B**) dopamine and (**C**) 5-hydroxytryptamine of ACh responses recorded under current-clamp conditions from the cockroach 'fast' coxal depressor motoneurone (D_f). ACh was applied locally to the neurone soma from a micropipette using brief pressure pulses delivered at 60 sec intervals. Each response is visible as a vertical line on traces (Ai), (Bi) and (Ci) (vertical scale 5 mV; horizontal scale, 2 mins); each amine reversibly reduces the amplitude of ACh responses. Selected ACh responses (marked with asterisks) are shown on an expanded time scale in (Aii), (Bii) and (Cii) (horizontal scale, 3 s). Taken from Fig. 9.1. Butt and Pitman (2002) (Wiley-Blackwell)

with pirenzepine. It is clear that the amines do not cause suppression of ACh responses by way of an increase in membrane conductance similar to that responsible for classical synaptic inhibition, since ACh currents recorded under voltage-clamp conditions are suppressed to a similar extent as are ACh-evoked changes in membrane potential observed in current clamp recordings (Fig. 9.10). These observations indicate that biogenic amines actually reduce the current flowing through the nicotinic ACh ion channel. In the case of 5-HT, this effect could be prevented by pharmacological antagonists of receptors for this amine (e.g. lysergic acid diethylamide (LSD) or RS23597), thus demonstrating that the action of 5-HT is receptor-mediated. However, attempts to characterize receptors mediating the actions of the amines were unsuccessful, since classical pharmacological antagonists frequently exerted agonistic effects, mimicking the actions of amines on ACh responses and rendering analysis impossible. Evidence that the effect of 5-HT is not produced primarily by a direct interaction of the amine with the nicotinic receptor itself

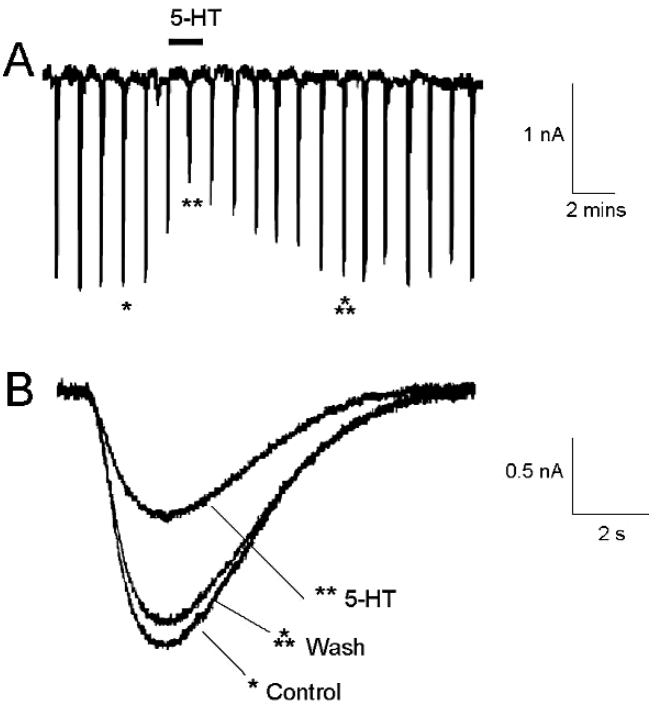

Fig. 9.10 5-Hydroxytryptamine (5-HT) suppresses nicotinic currents recorded from the cockroach 'fast' coxal depressor motoneurone (D_f) under voltage-clamp conditions. ACh was applied locally to the neurone soma from a micropipette using brief pressure pulses delivered at 60 sec intervals. (**A**) time-course of change in response amplitude caused by a brief bolus of 5-HT. (**B**) Selected ACh responses (marked with asterisks) shown on an expanded time scale. Taken from Fig. 9.4. Butt and Pitman (2002) (Wiley-Blackwell)

was provided by the observation that intracellular injection of GDP-β-S, (a GDP analogue that prevents activation of G-proteins) greatly reduced the ability of 5-HT to modulate ACh responses (Fig. 9.11). In fact, evidence indicates that 5-HT mediates its modulatory effects by way of cyclic AMP; the effects 5-HT are reduced by the adenylate cyclase inhibitor, dideoxyadenosine, and are mimicked by application of cyclic AMP or cyclic GMP analogues (Butt and Pitman, 2002, 2005). The effect of 5-HT on nicotinic ACh responses was prolonged when the phosphodiesterase inhibitor theophylline was applied to the preparation (Fig. 9.12). This effect would be expected if cyclic nucleotides mediate the action of 5-HT, since these intracellular messengers are normally broken down phosphodiesterase; their action would be prolonged, therefore, when this enzyme is inhibited. Protein phosphorylation is an essential step in the

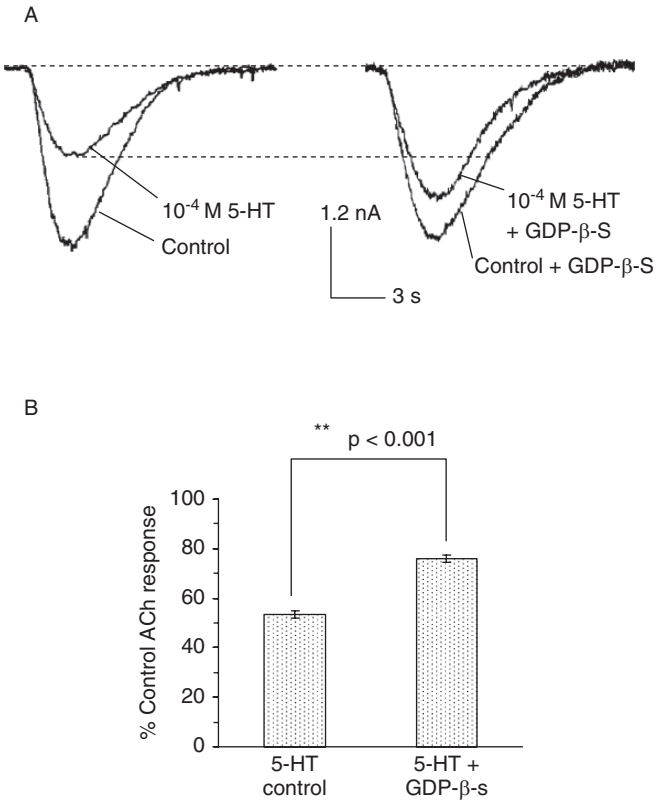

Fig. 9.11 Partial occlusion by GDP-β-S of the effect of 5-hydroxytryptamine (5-HT) on ACh-evoked currents recorded from the cockroach 'fast' coxal depressor motoneurone (D_f) under voltage-clamp conditions. (**A**) Intracellular injection of GDP-β-S reduces the extent to which ACh currents are modulated by 5-HT. (**B**) Bar graphs of pooled data showing that GDP-β-S significantly reduces the modulatory effect of 5-HT on ACh currents. Taken from Fig. 9.7. Butt and Pitman (2002) (Wiley-Blackwell)

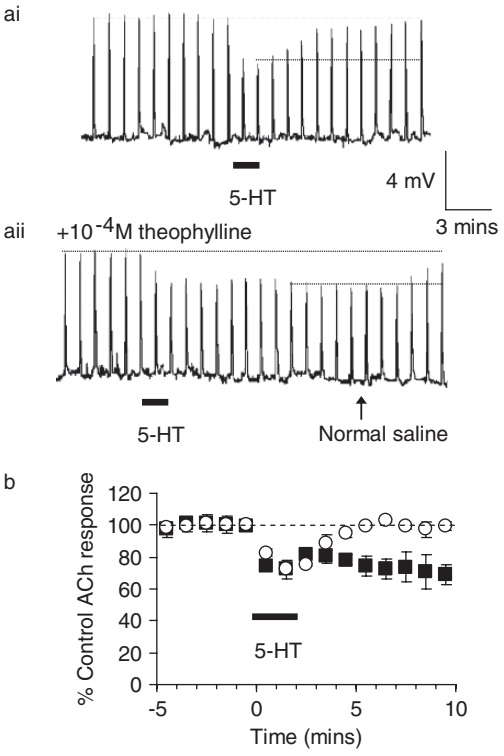

Fig. 9.12 The phosphodiesterase inhibitor theophylline prolongs the effect of a brief bolus of 5-hydroxytryptamine (5-HT) on ACh responses recorded from the cockroach 'fast' coxal depressor motoneurone (D_f) under current-clamp conditions. (**ai**) 5-HT causes a brief reduction in the magnitude of ACh responses. (**aii**) In the presence of theophylline, 5-HT-mediated suppression of ACh responses is greatly prolonged. (**b**) Data pooled from four experiments showing prolongation of the effects of 5-HT by theophylline (open circles, control; closed squares, in presence of theophylline). Taken from Fig. 9.3. Butt and Pitman (2005) (Wiley-Blackwell)

action of 5-HT, since 5-HT-mediated modulation of nicotinic responses was greatly suppressed by the broad spectrum protein kinase inhibitor, staurosporine, or by specific inhibitors of protein kinase A or protein kinase G (Fig. 9.13). Further support for a central role for phosphorylation in mediating the 5-HT-induced modulation of ACh responses is also provided by the observation that application of cantharidin, a phosphatase inhibitor, causes the action of 5-HT on ACh responses to become effectively irreversible (Butt & Pitman, 2005). This would be expected, since restoration of the ACh response amplitude after 5-HT wash-out would require dephosphorylation of the target protein. Thus, if phosphatase activity is blocked, the period of modulation would be prolonged. At present, the identity of the protein that is phosphorylated has not been established, although the simplest case would be that it is the nicotinic ACh receptor itself that is the target protein. Figure 9.14 summarizes the proposed pathways by which nicotinic signalling is modulated muscarinic receptors and by amine receptors.

The work summarized above highlights the potential for modulation of cholinergic sensory input to central neurones. Since the form of modulation described operates selectively upon ligand-gated ion channels, it could cause very selective effects. Muscarinic receptor-mediated modulation could be

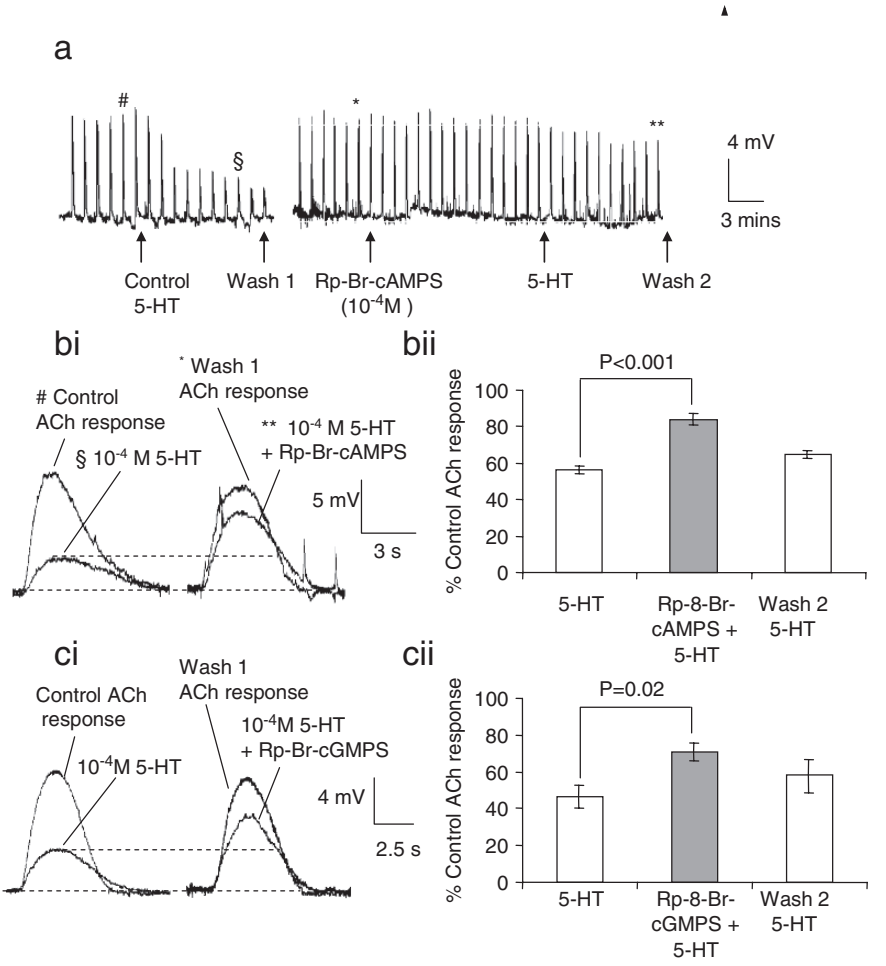

Fig. 9.13 Inhibitors of cyclic nucleotide-dependent protein kinases reduce the effect of 5-hydroxytryptamine (5-HT) on ACh responses recorded from the cockroach 'fast' coxal depressor motoneurone (D_f). (**a**) Recordings showing that application of the PKA inhibitor, Rp-Br-cAMPS (applied at arrow) reduces the effectiveness of a subsequent addition of 5-HT. (**bi**) Individual responses from (**a**) (marked with symbols) are shown on an expanded time-scale. (**bii**) Data pooled from 10 similar experiments, showing that Rp-Br-cAMPS has a significant effect. (**ci and cii**) Shows that the PKG inhibitor Rp-Br-cGMPS has a similar effect to that of Rp-Br-cAMPS. Taken from Fig. 9.5. Butt and Pitman (2005) (Wiley-Blackwell)

induced by sensory neurones themselves, perhaps being dependent upon the level or pattern of sensory activity. Under circumstances in which nicotinic signalling is suppressed at the same time as GABAergic signalling is enhanced, both changes would be synergistic and the overall effect could be one of reducing the responsiveness of central neurones. Since 5-HT, octopamine and

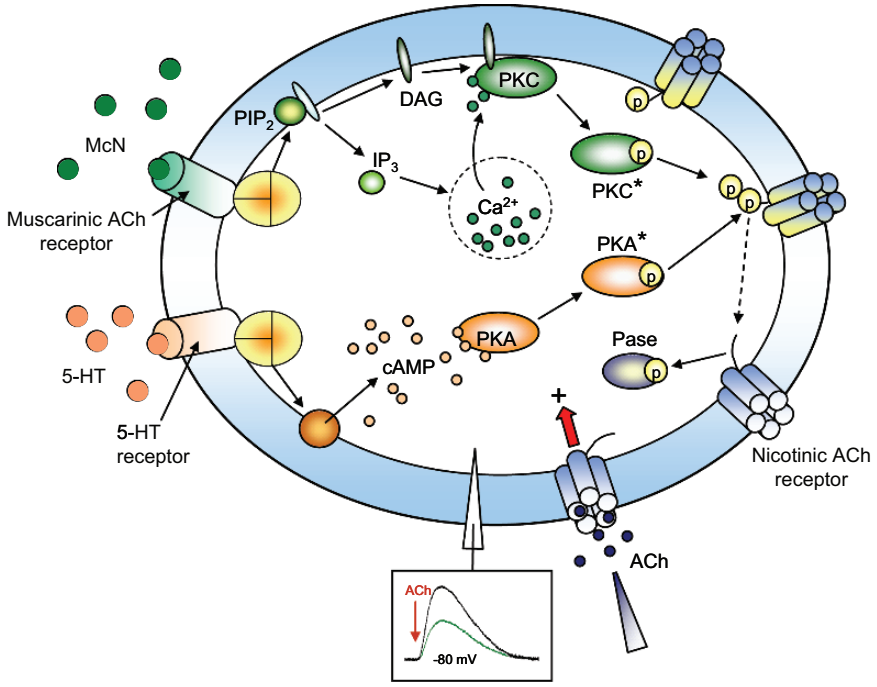

Fig. 9.14 Diagram summarizing the intracellular pathways by which muscarinic and amine receptors are proposed to mediate modulation of nicotinic ACh responses in the cockroach 'fast' coxal depressor motoneurone (D_f)

dopamine are primarily localized in interneurones rather than sensory neurones, aminergic modulation of cholinergic responses is more likely to be brought about as a result of centrally-generated state changes, which would, in turn, reduce the effectiveness of cholinergic inputs to neurones.

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Part III
Biomechanic of Nervous System

Chapter 10
Biomechanics of the CNS

Kristian Franze, Andreas Reichenbach and Josef Käs

Abstract For a long time, neurosciences have focused on biochemical, molecular, and electrophysiological aspects of cell functioning. However, there is an increasing awareness of the importance of biomechanics in physiology and pathology of the central nervous system (CNS). In the first part of this review we provide physical basics necessary to understand biomechanical measurements, we introduce the cytoskeleton as a major contributor to a cell's passive and active mechanical behavior, and we discuss some of the methods nowadays used to quantify mechanical properties. In the second part we present actual data on CNS mechanics, and we discuss the impact of passive mechanical material properties and active mechanical behavior of cells on the development, normal functioning and pathology of the CNS.

Keywords Biomechanics · viscoelasticity · elasticity · mechanical properties · stiffness · neurons · glial cells · tension · mechanosensitivity · cytoskeleton · AFM · SFM · optical stretcher

10.1 Introduction

The CNS is probably the most fascinating, complex, and still enigmatic organ system of our body. The first report about the nervous system was written on papyrus about 1700 years B.C., and about 1300 years later Hippocrates stated that the brain is the seat of intelligence. Since that time much progress has been achieved in understanding the morphology, biochemistry, and (electro-) physiology of nerve cells including their wiring and interactions. By contrast, the current knowledge about the biomechanical – i.e., viscoelastic – properties of these cells is rather limited. As will be detailed in Section 10.4, this is mainly due to the fact that only recently suitable methods were developed that allowed the

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A. Kamkin, I. Kiseleva (eds.), *Mechanosensitivity of the Nervous System*,

measurement of these properties. We want to point out here that CNS cells are confronted with very special environmental conditions, and that much of their development, functioning, and pathology cannot be properly understood without knowledge about their biomechanics.

Whereas we are used to think about vertebrates (and ourselves) as animals with an internal skeleton, our CNS is encapsulated by an external skeleton, similar to crabs or insects. This design provides protection against external mechanical impacts but also limits growth and expansion of the CNS during embryonic development or for example in case of edema. The velocity and duration of CNS tissue growth is different from that of its "exoskeleton", which contributes for instance to the formation of the gyri and sulci of the brain and to the formation of the cauda equina in the spinal cord (see Section 10.6.1). Throughout postnatal life, the cells in brain (encapsulated by the skull) and retina (encapsulated by the sclera) are under a pressure of some 10 mm Hg (Czosnyka and Pickard, 2004; Martin, 1992). This pressure may increase dramatically in cases of edema. While transient edema is not a serious problem for organs such as muscles or the liver, it becomes quickly deleterious for the CNS within its exoskeleton (Langfitt, 1969; Miller, 1975). One further issue during CNS development is the growth of neurites, which are the elongating cell processes of newborn neurons, through a complex environment consisting of tissues of varying stiffness. On the one hand, growing neurites may thus be confronted with obstacles in their way; however, on the other hand, the specific biomechanical properties of these tissues may be used by the neurites as cue that determines velocity and orientation of their growth (cf. Section 10.6.1).

The CNS not only consists of nerve cells (or neurons). In 1856, the German pathologist Rudolf Virchow, who was searching for a "connective tissue" in the brain, discovered a ubiquitous, non-neuronal cell type which he termed *glial cells* ("Nervenkitt"; the Greek word γλια means glue) (Virchow, 1856). He thereby ascribed these cells an exclusively mechanical function, namely to glue the neurons together. However, the mechanical properties of these cells remained completely unknown for the next 150 years (Lu et al., 2006). In addition to these cellular constituents of the CNS, meninges and blood vessels may have considerable impact on the local biomechanics of CNS tissue, due to both their stiffness (e.g., falx cerebri, large arteries) and the pulsatile changes of the blood pressure.

Nowadays, the application of biomechanics contributes to an understanding of many processes that take place in our body such as cell movement (Brunner et al., 2006; Cinamon et al., 2001; Lee et al., 1999; Prass et al., 2006), cell division (Grill et al., 2003; Matzke et al., 2001), phagocytosis (Kress et al., 2007) and contractility (Griffin et al., 2004a; Tan et al., 2003). Therefore, biomechanics helps understanding the normal functioning of living organisms, to predict changes which arise due to alterations of their environment, and maybe also to propose methods of artificial intervention. Most biomechanical investigations have so far focused on systems where mechanics obviously plays an important role, such as the locomotor system (Finer et al., 1994; Ishijima et al., 1991),

the cardiovascular system (Kellermayer et al., 1997; Rief et al., 1997), and the lung (Alcaraz et al., 2003; Wirtz and Dobbs, 2000). First measurements of mechanical properties of brain tissue have been performed at the end of the sixties (Fallenstein et al., 1969; Ommaya, 1968; Shuck et al., 1970); however, research started only very recently at the level of individual neurons or glial cells. This chapter is devoted to introduce physical basics of cell mechanics, to summarize these recent data, and to discuss its possible future impact.

10.2 Viscoelasticity

The mechanical properties of a material can be measured by applying strain, thereby deforming it, and then quantifying the resultant stress or by applying stress and quantifying the resultant strain. The study of the behavior of continuously deformable materials is termed *rheology*. Most materials will deform when an external load is applied. This deformation depends on their intrinsic properties and on their geometry. It is possible to classify materials with respect to their stress-strain relationship.

If the material is elastic, i.e., the applied energy is stored in the material, the resultant stress is proportional to the applied strain and vice versa, similar to the behavior of a spring (Janmey and Weitz, 2004). In case of such an elastic solid, the stress is exclusively a function of strain. The relation between stress and strain for linear elastic solids is shown in Fig. 10.1. The proportionality constant that relates stress to strain for a linear elastic solid is a measure for the resistance of the material to deformations. The higher this constant, the more elastic is the material, and the harder it is to deform.

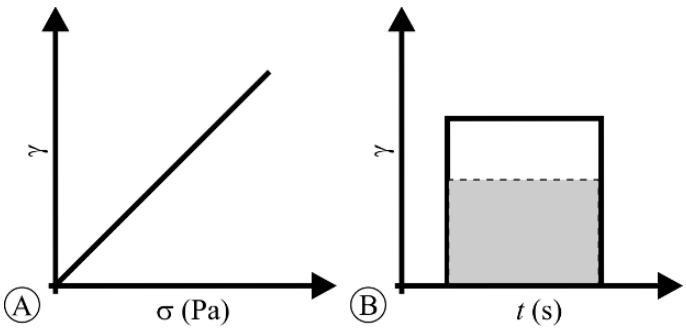

Fig. 10.1 Behavior of a linear elastic solid.
(**A**) For linear elastic materials, the stress σ is a function of the strain γ. The proportionality constant, e.g. spring constant, relates stress to strain and is a measure of the material's resistance to mechanical deformation. (**B**) Under a given stress (which is indicated as grey rectangle), the material deforms instantaneously proportionally to the load to a constant strain and elastically stores the applied energy. When subsequently the stress is removed, the sample recovers immediately to its original shape

In the simplest case of (Hookian) linear elastic deformation, the relationship between stress

$$\sigma = \frac{F}{A},$$

where F is the force and A is the cross-sectional area, and strain

$$\gamma = \frac{\Delta l}{l},$$

with Δl being the extension and l the natural length of the material, can be described by $\sigma = C\gamma$, where C is a tensor of elastic moduli (Fung, 1993).

In praxis, the strength of such a material can be investigated by applying a uniaxial deformation perpendicular to its surface. For linear, homogeneous, isotropic materials the stress here changes proportionally with the strain and can be related to the strain by the Young's modulus

$$E = \frac{\sigma}{\gamma}.$$

Alternatively, the material can also be sheared parallel to its surface. The (material) constant that in this case relates shear stress $\sigma = F/A$ to shear strain $\gamma = \Delta l/h$ (Fig. 10.2) is the shear modulus $G = \sigma/\gamma$ (Mezger and Zorll, 2000).

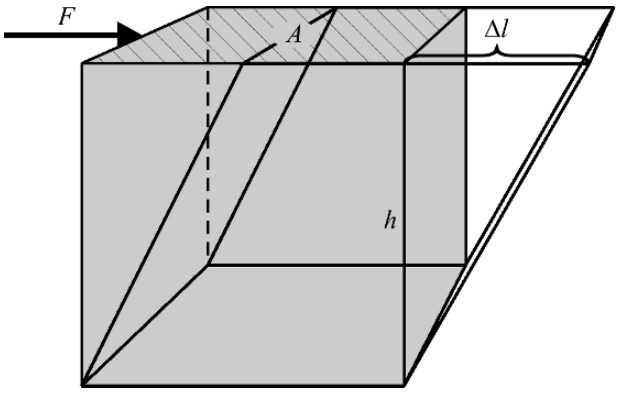

Fig. 10.2 Shear stress and strain.
When shear forces F act on the upper face of a cube of material (parallel to it) whose base is fixed to a surface, the cube will deform to an extend Δl. The shear stress is defined as F/A, where A is the upper (hatched) surface, shear strain is defined as deformation Δl/height h

For an isotropic, homogeneous material, the shear modulus relates to the Young's modulus by the Poisson's ratio ν as follows:

$$G = \frac{E}{2(1+\nu)}.$$

The Poisson's ratio is a material constant that describes the material's reduction of cross-sectional area as a consequence of its increase in length during a tensile test. It is defined as the ratio between relative change in thickness d to relative change in length l:

$$\nu = -\frac{\Delta d/d}{\Delta l/l}.$$

For most materials, ν assumes values between 0 and 0.5.

In contrast to elastic solids, in viscous fluids, which dissipate the applied energy by viscous flow, the resultant stress is proportional to the rate of strain or to how fast the material is deformed. In the simplest case of (Newtonian) linear viscous fluids, the relationship between the shear stress σ, which is a function of time t, and the strain rate $d\gamma/dt$ can be expressed as

$$\sigma(t) = \eta \frac{d\gamma(t)}{dt},$$

where η is the coefficient of viscosity (Fig. 10.3).

However, Newtonian fluids and Hookian elastic solids are abstractions which are barely found in nature. Some materials may follow these laws within certain boundaries. Nevertheless, most real materials show a more complex behavior. In particular, many materials combine both solid- and fluid-like behavior. For those *viscoelastic* materials, such as biological tissues, the relationship

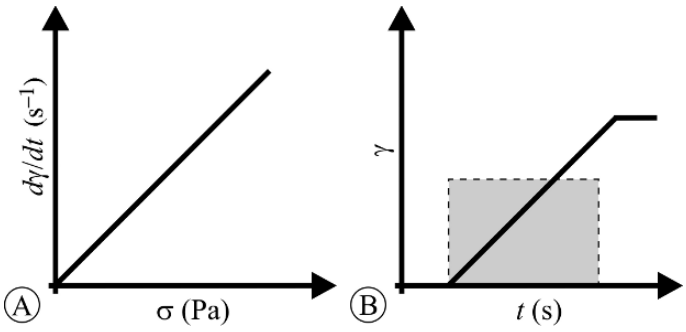

Fig. 10.3 Behavior of a linear viscous fluid.
(**A**) For linear viscous materials, the stress is a function of strain rate. The proportionality constant is a measure of the material's viscosity. (**B**) If a constant stress is applied to a viscous material (which is indicated as grey rectangle), it will flow at a constant rate, which is proportional to the load, and viscously dissipate the applied energy. When subsequently the stress is removed, the flow ceases and the material remains in its deformed state

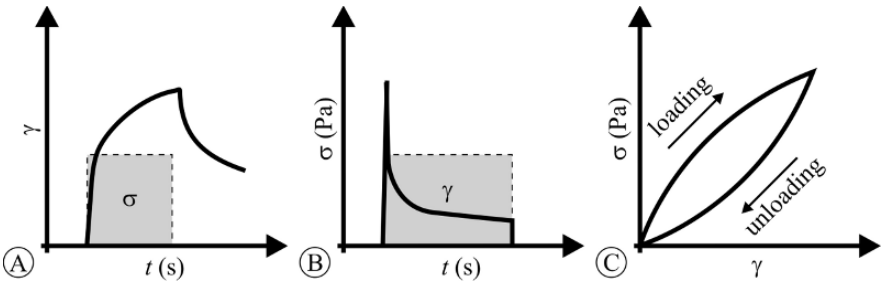

Fig. 10.4 Behavior of viscoelastic materials.
(**A**) Creep: when a constant stress is applied to a viscoelastic material, its deformation increases nonlinearly over time. Thereby, the immediate response is dominated by the elastic component, while on longer time scales viscosity is dominant. When the stress is removed, the material recovers partly over time, the more elastic the material the higher the recovery. (**B**) Stress relaxation: when a constant strain is applied to such a material, the stress in the material decreases nonlinearly over time. While viscoelastic fluids totally relax after a certain time, viscoelastic solids relax to a finite value. (**C**) Hysteresis: If viscoelastic materials are subject to cyclic loading, the stress-strain relationship in the loading process differs from that in the unloading process

between stress and strain changes with time. Here, the stress-response depends on both the strain applied and the rate at which it is applied (Fig. 10.4).

A viscoelastic material is characterized by three typical responses to external loads. When a constant stress is applied to such a material, its deformation will increase nonlinearly over time. This behavior is called *creep* and is for example seen in typical experiments in which cells are deformed with an optical stretcher (Guck et al., 2000; Wottawah et al., 2005). On the other hand, when a step strain is applied to this material, the stress will decrease nonlinearly with time. This *stress relaxation* is also known to be typical for biological tissues and cells (Charras and Horton, 2002). The stress relaxation modulus

$$G(t) = \frac{\sigma(t)}{\gamma}$$

is defined as the ratio between the stress σ remaining at time t and the magnitude of the step strain γ (Rubinstein and Colby, 2003). Finally, the internal friction of a viscoelastic material leads to *hysteresis*, which refers to a lagging of an effect behind its cause. Hysteresis represents the history dependence of physical systems that do not instantly follow applied forces, but react slowly or do not return completely to their original state (Fung, 1993).

Because the mechanical response of a viscoelastic material is a function of load magnitude and rate, both must be specified during an experimental study. There are two typical ways to determine the mechanical properties of a viscoelastic material: dynamic and static experiments. Dynamic methods involve the application of harmonically varying stresses or strains. Static methods involve

the imposition of a step change in stress (creep experiments) or strain (relaxation experiments) and the observation of the subsequent development of the strain or stress as a function of time.

Applying a specified sinusoidally oscillating strain or stress to a material enables the direct determination of its load and frequency dependent mechanical properties (Schramm, 1994). In this case, the material is for example subject to a periodical strain γ at an angular frequency ω with $\gamma = A\cos(\omega t + \varphi)$, where A is the amplitude and φ is the phase angle. γ can be considered the projection of a rotating vector on the real axis. Because a vector is specified by two components, it can be represented by a complex number. Thus, the vector $\gamma(t)$ can be described by its two components $\gamma'(t) = A\cos(\omega t + \varphi)$, which represents the real part, and $\gamma''(t) = A\sin(\omega t + \varphi)$, which represents the imaginary part. Therefore, $\gamma(t)$ can be described by the complex number $\gamma^* = \gamma' + i\gamma''$, where $i = \sqrt{-1}$. In analogy, the stress σ measured during a dynamic mechanical analysis can be described as $\sigma^* = \sigma' + i\sigma''$.

For a linear viscoelastic material, the amplitude of stress is proportional to that of strain, and the stress alternates sinusoidally at the same frequency, but it is out of phase with the strain, i.e., there is a lag between the applied strain and the resultant stress, which defines the *phase angle* δ (Fig. 10.5). In case of ideal elastic materials, the phase angle is 0°, i.e., stress and strain are in phase, so that the response of stress caused by the applied strain is immediate. In contrast, in purely viscous materials, stress and strain are out-of-phase, the phase angle is 90° (Davis, 1974). Consequently, in viscoelastic materials, the phase angle between the applied strain (or stress) and the resulting stress (or strain) is between 0° and 90°.

During a dynamic rheological experiment, the phase angle and the ratio of the amplitudes of stress and strain are measured and can be used to calculate

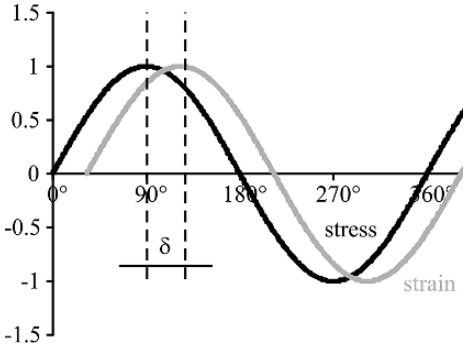

Fig. 10.5 Sinusoidal testing of shear properties.
When a stress or strain, whose value is changing continuously according to a sine wave, is applied to a viscoelastic material, the induced response (strain or stress) will also follow a sine wave at the same frequency, but is out of phase. By measuring the ratio of stress to strain and the phase difference δ between the two, storage and loss moduli can be calculated

the parameters which represent viscoelastic behavior. Here, the complex shear modulus, $G^* = G' + iG''$ (in case of stresses that act parallel to the object's surface), or the complex Young's modulus, $E^* = E' + iE''$ (in case of stresses that act normal to the object's surface), represent the total mechanical resistance of a material against deformation. These complex moduli are separated in an in-phase (real) and an out-of-phase (imaginary) component. The real part, or *storage modulus*,

$$G' = G * \cos\delta = \frac{\sigma_0}{\gamma_0}\cos\delta,$$

where σ_0 and γ_0 are the amplitudes of stress and strain and δ is the phase shift between them, reflects the response of the material that is in phase with the applied oscillatory deformation. Therefore, it is a measure of the elastic energy stored by the sample during the deformation. The imaginary part, or *loss modulus*,

$$G'' = \frac{\sigma_0}{\gamma_0}\sin\delta,$$

reflects the material's response out of phase with the oscillatory deformation and is a measure of the viscous dissipation of energy. In other words, the storage modulus is a measure of a material's elastic stiffness and the loss modulus measures its viscous properties. The dimensionless loss tangent

$$\tan\delta = \frac{G''}{G'}$$

is a measure to which extent an object is elastic ($\delta \approx 0$) or viscous ($\delta \approx 90^\circ$). The smaller the loss tangent, the more elastic is the material (Davis, 1971).

10.3 The Cytoskeleton

Whether a material stores externally applied kinetic energy or dissipates it depends on its internal structure. The inherent biomechanical properties of cells determine their shape and how they are deformed when subjected to mechanical forces. The structure responsible for these inherent mechanical cellular properties is the cytoskeleton (Bursac et al., 2005; Rotsch and Radmacher, 2000; Wang et al., 1993). The cytoskeleton is a dynamic structural and functional framework that is made up of a complex entangled, transiently cross-linked polymer network. This network is composed of three major types of biopolymers and of associated proteins such as motor proteins and cross-linkers (Alberts et al., 2002).

It is possible to describe this biopolymer network in a general way in terms of polymer physics. Thereby, the bending modulus of the different filaments can be used to determine the mechanical properties of the cytoskeleton. Deforming a straight filament requires the bending energy

$$E_{bend} = \frac{1}{2}\kappa \int \left(\frac{\partial \vec{t}_s}{\partial s} \right)^2 ds,$$

where κ is the bending modulus and $\vec{t}_s$ is the tangent vector of the filament contour, parameterized by the contour length s (Kratky and Porod, 1949).

Because biopolymers are subject to thermally driven fluctuations, the bending modulus can be related to the thermal energy. The resulting persistence length

$$L_p = \frac{\kappa}{k_B T}$$

is an important parameter defining the flexibility of a polymer chain. This parameter can be used to distinguish three different classes of polymers, which can be modeled using different theories (Doi and Edwards, 1988). If the actual length of the filaments is small compared to their persistence length, they are considered rigid rods. If the total filament length is in the same range as the persistence length, the polymer is semiflexible. And if the persistence length of the filament is small compared to its actual length, the filament is called flexible.

Microtubules are hollow cylindrical structures, which constitute one of the three main types of cytoskeletal filaments. With a length-dependent persistence length of hundreds of micrometers to some millimeters (Pampaloni et al., 2006) they are considered rigid rods. Their diameter is about 25 nm. Microtubules possess a polarized structure with a (+) and a (−) end. Amongst others, they are main contributors to mitotic spindle assembly and function (Bringmann et al., 2004), and they are also crucial in neuronal migration (Schaar and McConnell, 2005). They exhibit two mechanistic classes of interactions: regulatory and structural – together with actin, the second polymer class of the cytoskeleton (Rodriguez et al., 2003).

The actin network is composed of semiflexible polymers with a persistence length of ~9 μm (Isambert et al., 1995), which is in the range of a cell's size. Actin filaments have a diameter of about 7–9 nm. They also possess a polarized structure with a barbed and a pointed end. The submembranous actin cortex is thought to be the main contributor to a cell's mechanical properties and, thus, to its shape and motility (Ananthakrishnan et al., 2006; Gardel et al., 2004). However, there is also evidence that in some cases other compartments of the cytoskeleton are at least equally if not more important for a cell's mechanical behavior (Charras and Horton, 2002; Hoffman et al., 2006).

The third cytoskeletal class consists of intermediate filaments, which are flexible, apolar polymers with a persistence length of ~1 μm (Mucke et al., 2004). They obtained their name because of their diameter of about 10–12 nm, which is right between those of microtubules and actin filaments. Amongst others, they are thought to be important for the resistance to mechanical stresses in epithelial and muscle cells as well as in Müller cells (Lundkvist et al., 2004), and they regulate astrocyte motility (Lepekhin et al., 2001). Some intermediate filaments are ubiquitous (e.g., vimentin), while others are restricted to specific cell types. For instance, neurons contain neurofilaments and most astrocytes in the brain contain glial fibrillary acidic protein (GFAP). The expression of intermediate filaments in macroglial cells changes during pathological events (Lewis and Fisher, 2003; Pekny et al., 1999). The reason for this alteration of the cytoskeleton is not yet fully understood.

All three classes of biopolymers harden when they are strained (Janmey et al., 1991). While actin filaments and microtubules break already at comparatively low strains, intermediate filaments resist breakage and may thus be crucial for maintaining cell integrity in case of high deformations (Fuchs and Cleveland, 1998; Wang and Stamenovic, 2000).

The cytoskeleton is not just a passive polymer network. Rather, it is subject to continual active reorganization processes. Polymer filaments are incessantly assembled and disassembled (Nedelec et al., 1997), cross-linkers, which are proteins that link different cytoskeletal polymers to each other, are continuously attaching to and detaching from the polymers, and molecular motors are also actively contributing to the cells' mechanical properties. This dynamic equilibrium of all cytoskeletal components, which is tightly controlled by the cells, allows immediate adaptations to changes in their environment (Wakatsuki et al., 2001; Wang et al., 2001).

Even more, the whole cytoskeleton is linked to the cells' environment, i.e., to the extracellular matrix and to other cells, particularly by connections through intramembranous adhesion molecules such as integrins and cadherins (Cavalcanti-Adam et al., 2006; Wang and Ingber, 1994). The coupling of the cytoskeleton to the extracellular matrix allows active cellular mechanosensitivity. Cells can probe the mechanics of their environment and respond to it, an event termed "mechanotransduction" (Discher et al., 2005; Flanagan et al., 2002; Georges et al., 2006; Maniotis et al., 1997; Potard et al., 1997; Wang et al., 1993; Wang and Ingber, 1994; Yeung et al., 2005). As a consequence, cells regulate their shape and motility according to the mechanics of their neighborhood (Bischofs and Schwarz, 2003). To probe the mechanical properties of their substrate, cells adhere to it and deform it (Franze et al., 2008; Munevar et al., 2001). At the same moment, the surrounding exerts forces on the cells, thus *prestraining* them (Ingber, 2006). The resulting cellular *prestress* in turn is important for the cells' rheological properties (Stamenovic et al., 2002).

Finally, the cytoskeleton may be disturbed in different diseases such as malaria (Suresh et al., 2005) or in certain types of cancer (Remmerbach

et al., 2008), which may result in altered mechanical properties of the concerned cells.

10.4 Measurement Techniques

The mechanical properties of biological samples can be measured on different length scales. Viscoelastic properties can be obtained from structures as large as tissue slices (centimeters) down to subcellular structures (nanometers).

A standard tool for the investigation of mechanical properties of bulky samples such as tissue slices is a rheometer. In a rheometer, the tissue is mostly confined between two round and parallel surfaces (e.g., plates) that can be moved relative to each other. A motor drives the lower plate in an oscillatory fashion at a range of particular frequencies, ω, and around a certain angle, θ, which depends on the specified (torsional) shear strain γ, the sample's thickness h, and the radius r of the geometry (Mezger and Zorll, 2000) (Fig. 10.6):

$$\theta = \frac{\gamma h}{r}.$$

The measurement of the corresponding oscillation is made on the upper plate, which is connected to the lower driven one by the sample (see Fig. 10.6). Therefore, the movement of this upper plate can be used to determine the stress that results from the applied strain. The stress that follows the motion of the strain arises from the solid-like response of the material. Thus, the elastic storage modulus $G'(\omega)$ can be obtained. The stress that is proportional to the speed of the applied motion arises from the fluid-like response of the material. Thereby,

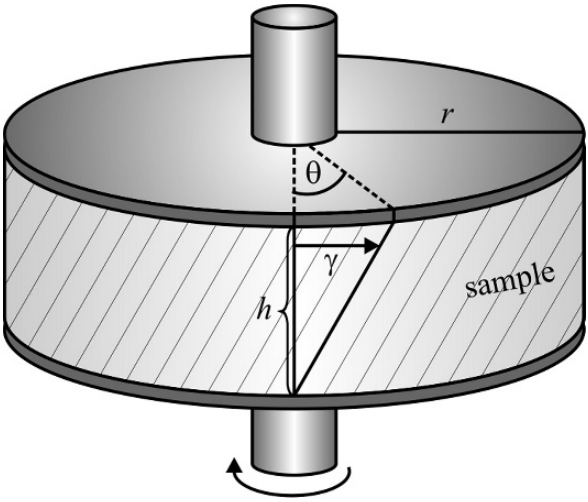

Fig. 10.6 Principle of a rheometrical measurement. An oscillatory shear strain $\gamma = \theta r/h$ is applied to a sample of height h by a rotating plate; the measurement is made on the other plate with radius r, which is connected to the driven one by the sample. The movement of this passively following plate, which is shifted compared to the driven plate by an angle θ can be used to determine the sample's rheological parameters

the viscous loss modulus $G''(\omega)$ can be obtained. Alternatively, and also depending on the rheometer used, a stress can be applied to the sample and the resulting strain can be measured to determine the sample's viscoelastic properties (Gehm, 1998).

For the measurement of mechanical properties of whole cells, some of the most widely spread methods include microplate manipulations (Thoumine and Ott, 1997) and micropipette aspiration (Evans and Kukan, 1984; Schmidschonbein et al., 1981). Apart from mechanical techniques also optical methods are exploited to characterize cellular mechanical properties (Ashkin et al., 1987; Kuo, 2001; Sterba and Sheetz, 1998; Svoboda and Block, 1994). An optical stretcher is a dual beam infrared laser trap which can stably trap and – at higher laser powers – deform whole suspended cells along the laser axis. The deformation arises from optical forces that act on the cell surface (Guck et al., 2001; Guck et al., 2000).

When light enters or exits a transparent dielectric body that has a different refractive index than its surrounding, it transfers a momentum to this body (Ashkin, 1970). If the refractive index n of the body is higher than that of the surrounding, which is true for biological cells in suspension ($n_{\text{water}} = 1.33$, $n_{\text{cell}} \approx 1.4$), the light with energy E gains momentum p when entering the cell:

$$p = \frac{nE}{c},$$

where c is the speed of light in vacuum. The beam of a laser exiting a single mode optical fiber diverges and features a Gaussian intensity distribution, i.e., its intensity decays radially. The transverse profile of the intensity I of a Gaussian beam with a power P can be described by

$$I(r,z) = \frac{2P}{\pi\omega(z)^2}\exp\left(-\frac{2r^2}{\omega(z)^2}\right),$$

where r is the distance radially from the beam axis, z the distance from the source fiber, and $\omega(z)$ the beam width, which is defined as the transverse dimension within which the electric field distribution of the beam decreases to a value of $1/e$ ($\sim$37%) of its maximum on the beam axis and within which $\sim$86.5% of the beam energy is contained.

Considering such a divergent laser beam incident on a cell, the conservation of momentum leads to forces that are normal to the cell surface and point away from the denser medium (Ashkin, 1970; Casner and Delville, 2001; Guck et al., 2000). Due to the reflection of a fraction of the light on both surfaces (entrance and exit) and a collecting lens effect of the cell itself, which focuses the light, the forces that point away from the laser source are higher than those in opposite direction. This leads to the so-called scattering force which pushes the cell away from the laser source. Since a laser beam emanating from a single

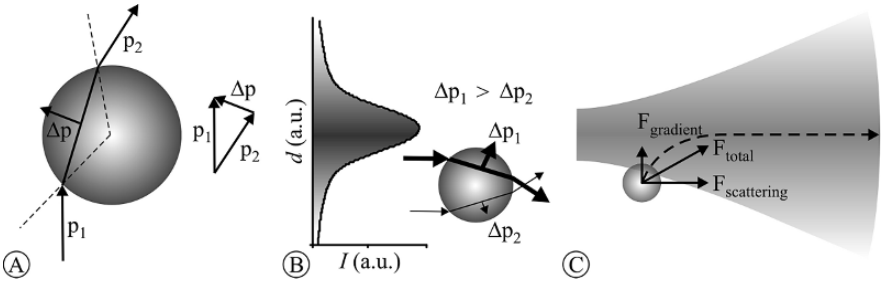

Fig. 10.7 Principle of a dual beam laser trap.
(**A**) When light enters or exits a transparent dielectric object with a refractive index different from its surrounding, the conservation of momentum *p* leads to forces that act away from the denser medium and normal to its surface. (**B**) Because of the Gaussian intensity profile of a laser beam that emanates from a single mode optical fiber (*left*), any object with a higher refractive index than that of its surrounding will be moved to the laser axis where the intensity is highest (*right*). (**C**) This gradient force together with the scattering force, which pushes the object away from the laser source, cause the object to be moved as indicated by the dashed arrow. Introducing a second, counter-propagating laser of the same power creates a stable trap, since the sum of scattering forces in the trap's center is zero

mode optical fiber features a Gaussian intensity gradient, the cell is additionally pulled towards the center of the beam because of the higher laser intensity (Fig. 10.7). Thus, by introducing a second, counter-propagating divergent laser beam of the same power, the scattering forces can be balanced and, consequentially, the cell is stably trapped between the two beams. A further increase of laser power results in a stretching of the cells along the laser axis (Guck et al., 2001; Guck et al., 2000; Wottawah et al., 2005). Thus, with the optical stretcher cells can be probed without any mechanical contact, contributing to a very gentle treatment and allowing the investigation of even extremely fragile cells.

Since cells are composed of a variety of different compartments, their mechanical properties differ from cellular region to region (Hoffman et al., 2006; Lu et al., 2006). For the investigation of those mechanical heterogeneities a whole set of techniques is available. Many of them either use nanobeads, which are embedded into the cells or attached to their surface, or indent the cells at different locations. In the former case, the beads may either be passively observed and their trajectories are analyzed ("particle tracking") (Crocker and Hoffman, 2007; Tseng et al., 2002) or they are actively used to exert forces to the cells ("magnetic bead twisting", "optical tweezers") (Bausch et al., 1998; Svoboda et al., 1992). Among indentation techniques, cell poking (Mcconnaughey and Petersen, 1980) and scanning force microscopy belong to the most widely spread ones.

The scanning or atomic force microscope (SFM or AFM) (Binnig et al., 1986) is a mechano-optical instrument which is used to image samples with nanometer resolution (Henderson et al., 1992) and to measure forces in the

piconewton range (Mahaffy et al., 2000; Radmacher, 1997). Unlike electron microscopes, it can image and probe samples in air or in liquid. Thus, it is possible to investigate living cells in real time (Lu et al., 2006; Moreno-Herrero et al., 2004; Radmacher et al., 1992).

The core of an SFM is a soft elastic leaf spring, the cantilever. At its end, a pyramid shaped sharp tip is mounted perpendicular to the cantilever axis. Additional to the cantilever, an infrared laser and a four-quadrant photodiode comprise the scanning unit. The laser beam is reflected at an oblique angle from the very end of the cantilever to the position-sensitive photodiode. The scanning unit can be moved in three dimensions by a set of piezoelectric elements (Fig. 10.8). A computer is connected via a signal access box to the photodiode and the piezos (Alonso and Goldmann, 2003; Dufrene, 2002). When the tip is brought into proximity of the sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law $F = k\Delta x$, where F is the force, k is the spring constant of the cantilever, and Δx is the cantilever's deflection. The deflection causes a change of the reflected laser's position on the photodiode, which can be measured.

The compliance of a sample area can be determined by deforming it with the cantilever. The scanner raises the sample or lowers the cantilever by a preset amount, the "modulation amplitude". In response, the cantilever deflects gradually depending on the stiffness of the sample: the harder the sample, the more the cantilever deflects (Fig. 10.9). The velocity of the cantilever and the indentation depth, which depends on the applied force and the sample's compliance, can be controlled. The height information of the z-piezo and the deflection of the cantilever are recorded during such an experiment, resulting in typical *force-distance curves*. These curves are used to calculate the sample's mechanical properties (see below).

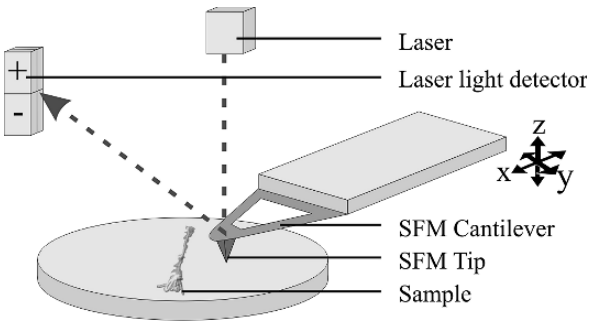

Fig. 10.8 Functional principle of an SFM.
A soft leaf spring ("cantilever") mechanically interacts with a sample. Attractive or repulsive forces lead to deflections of the cantilever. The magnitude of deflection is captured by a laser beam that is reflected from the very end of the cantilever to a four quadrant photodiode. In scanning mode, the cantilever's deflection provides topographical images of the sample's surface, in spectroscopy mode the deflection depends on the material's resistance to deformations and thus provides information about its mechanical properties

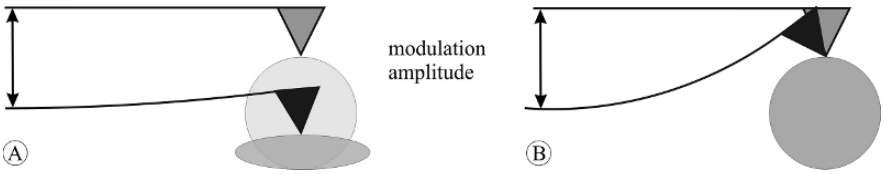

Fig. 10.9 Principle of the force measurement with an SFM.
When the cantilever is lowered towards the sample by a preset amount ("modulation amplitude"), the cantilever indents the sample and deflects. The modulation amplitude corresponds to the sum of cantilever deflection and indentation. The stiffer an object, the less it is deformed and the more the cantilever deflects. This information can be used to calculate the sample's elasticity

Different approaches can be exploited to determine the exact position of a force measurement on a cell. For instance, cells can be scanned prior to the measurement. Depending on the sample, its environment and the aim of the study, different scanning modes can be exploited. Commonly used are either static (contact) or dynamic modes. In any case, the sample has to be well adhered to the substrate, otherwise the cantilever would push it away while scanning the substrate (El Kirat et al., 2005). The adherence of acutely isolated cells is mostly too low to achieve good images (Rosenbluth et al., 2006). A further disadvantage of the mechanical contact between the cantilever and the cells during a scan is the possible impact on the cytoskeleton; active cellular responses to this mechanical stimulus may be triggered (Deng et al., 2004; Smith et al., 2003; Stamenovic et al., 2002). The position of a force measurement can also be determined – and the (mechanical) scanning be avoided – by combining scanning force microscopy with optical microscopy techniques, such as phase contrast, epifluorescence or confocal laser scanning microscopy.

In force measurements, optimal signal-to-noise ratios are obtained when the cantilever's deflection is in the same range as the indentation of the sample. Typical cantilevers used with living samples have therefore spring constants of about 0.01–0.15 N/m. Standard cantilever tips are pyramidal shaped with a contact area of about 20 nm diameter, which allow spatially high resolved measurements. To avoid damage of cells and to obtain a well defined contact area between cantilever probe and the sample, cantilever tips may be modified. For example, polystyrene beads of different diameters can be glued to them (Mahaffy et al., 2004; Mahaffy et al., 2000). To evaluate the modified cantilever probe, its surface can be scanned by gluing a second cantilever upside-down to a glass slide and scanning its sharp pyramidal shaped tip with the modified cantilever. The resulting image allows an assessment of the probe's surface quality as well as an accurate determination of its radius R (Fig. 10.10). From

$$R^2 = \left(\frac{d}{2}\right)^2 + (R-h)^2 \text{ follows } R = \frac{4h^2 + d^2}{8h}.$$

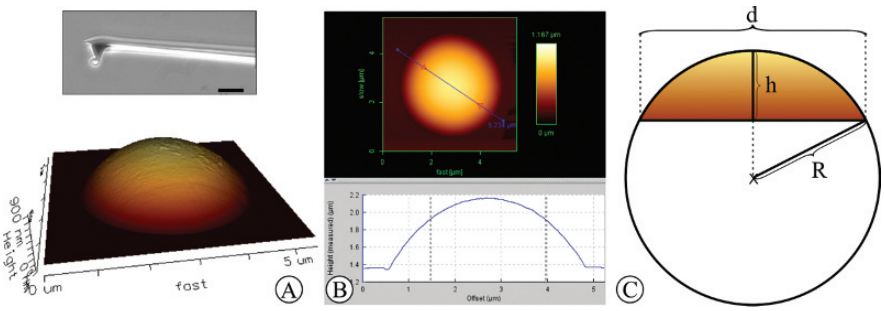

Fig. 10.10 Characterization of modified cantilevers.
(**A**) The beads, which are glued to the cantilever tips (*upper part*, Bar: 20 µm, image courtesy of Jens Gerdelmann) are characterized in terms of surface quality, position on the cantilever to which they are attached, and radius. *Bottom*: The bead's shape and surface are of good quality. (**B**) The scanned image (*upper part*) is used to determine the exact bead radius. *Bottom*: A cross-section (line in the *upper part*) is drawn through the bead's center and can be used to measure the quantities *d* and *h*, which are illustrated in figure (**C**), whose upper section represents the part of the bead which is scanned

To extract the mechanical properties of the sample from force-distance curves, the cantilever's spring constant has to be determined and the photodiode has to be calibrated. For the calibration, force distance curves are taken on a hard surface, e.g., on glass slides. Here, the cantilever deflection equals the *z*-piezo movement since the indentation of the substrate is negligible. The slope of the resulting curves reveals the sensitivity

$$S = -\frac{1}{slope}.$$

The determination of the cantilever's spring constant is a known error source (Cumpson et al., 2004). The calculation of the spring constants from the geometry of the cantilevers provides relatively inaccurate results. Pushing the cantilever against a second cantilever with known spring constant $C_{cantilever}$ is a more reliable solution. The spring constant of the unknown cantilever $C_{unknown}$ can be calculated with

$$C_{unknown} = C_{cantilever}\left(\frac{1}{S_{underground}} - \frac{1}{S_{cantilever}}\right) * S_{cantilever},$$

where $S_{underground}$ is the sensitivity of the unspecified cantilever when pushed against an infinitely hard surface and $S_{cantilever}$ is its sensitivity when pushed against the specified cantilever. Nevertheless, the reference cantilevers are usually obtained from commercial providers, and the reliability of their specifications is often questionable. One of the most reliable techniques nowadays available to approximate a cantilever's spring constant is a technique called

"thermal noise" (Hutter and Bechhoefer, 1993). Here, thermal fluctuations in the cantilever's deflection are measured. The equipartition theorem is then used to relate this to the spring constant. Basically, the thermal energy calculated from the temperature should be the same as the energy measured from the oscillations of the cantilever.

The mechanical properties of the sample can then be calculated from the force-distance curves using different mathematical models. The commonly used Hertz model is valid for small deformations of a thick sample of a linear elastic, isotropic, homogeneous material (Hertz, 1881). It describes the elastic behavior of two elastic spheres in contact and has been modified to include other shapes (Dimitriadis et al., 2002; Mahaffy et al., 2004; Mahaffy et al., 2000). The equation relating the force F exerted by a cantilever modified with a bead to the corresponding indentation δ of the cell is the following:

$$F = \frac{4}{3}\frac{E}{1-\nu^2}\sqrt{r}\delta^{3/2}.$$

Here, E is the cell's Young's modulus, ν is its Poisson's ratio, and r is the radius of the bead that is glued to the cantilever.

The data directly provided by a measurement is the displacement of the z-piezo Δz, and the vertical deflection of the cantilever Δc. The contact point *c.p.*, at which the cantilever first touches the cell, is the first point of the force-distance curve where its slope differs from zero. The indentation δ can be determined from these data with $\delta = \Delta z - \Delta c - c.p.$ Since the cantilever is a Hookian spring, the force acting on it is given by $F = k\Delta c$. The cantilever's spring constant k is calibrated and the radius of the bead can be determined as described above. Because both Young's modulus E and Poisson's ratio ν represent material constants, the elasticity constant K is defined as

$$K = \frac{E}{1-\nu^2}.$$

Thus, the cell's elasticity can be calculated using the following equation:

$$K = \frac{3}{4}\frac{k\Delta c}{\sqrt{r}(\Delta z - \Delta c - c.p.)^{3/2}}$$

However, the Hertz model is only valid for very small indentations compared to the sample size, i.e., when the sample's thickness is larger than 15 times the indentation depth (Engler et al., 2004c). In most cell measurements this is not feasible. Different correction terms, which account for substrate effects during the measurement of thin samples, have been introduced (Dimitriadis et al., 2002; Mahaffy et al., 2004). To correct for this substrate effect, knowledge about the sample height is required. This height can be inferred either from an SFM scan or, alternatively, it can be calculated by subtraction of the z-piezo

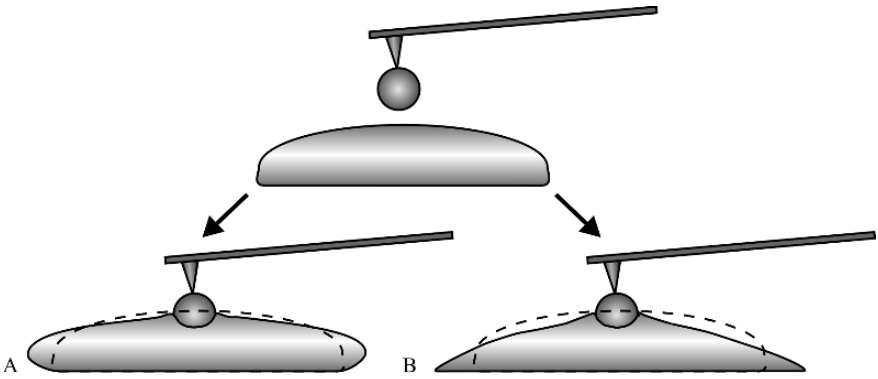

Fig. 10.11 Application of Tu and Chen models.
The Hertz model used to describe the elastic behavior of two spheres in contact mostly overestimates the elasticity of cells because of substrate effects. To include the contribution of the substrate to the measured values, the common Hertz model can be extended according to Tu and Chen. (**A**) The Chen model is valid for well attached cells, which are closely coupled to the substrate. If this model is applied, also the cell's Poisson's ratio can be obtained. (**B**) The Tu model reveals the actual elasticity values of non-adhered cells

position at the moment of first contact with the sample from the *z*-position of the underlying substrate. The latter can be calculated by fitting a plane through the contact points of three force-distance curves taken on the substrate around the sample (Lu et al., 2006).

One way to account for thin samples is the extension of the Hertz model by Tu and Chen models, which are correction terms that apply for non-adhered and well-adhered regions of the cells, respectively (Mahaffy et al., 2004) (Fig. 10.11). Because always only one of these models fits the data, additional information about the adherence of a cell and - in case of the Chen model – about the cell's Poisson's ratio can be obtained (Mahaffy et al., 2004).

Finally, the possibility of performing frequency dependent microrheology using a scanning force microscope is worth mentioning. The oscillatory drive signal necessary to perform these frequency-dependent viscoelasticity measurements can be fed to the scanner signal through a lock-in amplifier (Mahaffy et al., 2000). Amplitude as well as phase differences between the applied modulation and the cantilever response signal are then recorded.

10.5 Viscoelastic Properties of the CNS: Available Data

Over the last four decades numerous studies have substantiated the fact that nervous tissue is a nonlinear, viscoelastic material (Darvish and Crandall, 2001; Donnelly and Medige, 1997; Gefen and Margulies, 2004). Since its mechanical response to a deformation therefore depends on its total strain history, there are

many different ways to characterize its mechanical behavior. Even more, the immense progress in life sciences during the last decades was incessantly accompanied by enhancements, modifications, and improvements of existing methods as well as by the development of completely new measurement techniques. This development has led to more and more possibilities for the determination of parameters that describe the complex mechanical behavior of nervous cells and tissue.

An inevitable consequence of this diversity is the intricacy – and sometimes impossibility – to compare results collected with different methods. There is a large amount of data on mechanical properties of nervous tissue reported in literature that shows huge discrepancies even in the linear viscoelastic regime (Garo et al., 2007; Gefen and Margulies, 2004). Most studies on brain material properties have focused on short-duration loading using oscillatory shear tests, where the brain behaves relatively stiff. However, relevant time scales for strains exerted and experienced by individual cells *in vivo* are assumed to be rather in the range of seconds to hours (Discher et al., 2005). Long-term moduli of nervous tissue as for instance tested using stress relaxation measurements revealed a significantly softer characteristic (Bilston et al., 1997; Georges et al., 2006).

Depending on the methods used and also on experimental parameters such as species and age of the animal, postmortem time interval prior to testing, sample preparation, exact location of the measurement, strain magnitudes, or loading rates, shear moduli between few hundred Pa up to tens of kilopascals were measured for brain tissue (Gefen et al., 2003; Hirakawa et al., 1981; Kruse et al., 2008; Lu et al., 2006; Miller et al., 2000; Nicolle et al., 2005; Prange and Margulies, 2002). However, due to its nonlinear, time-dependent viscoelastic nature an exact quantification of the mechanical properties of the CNS is certainly difficult to accomplish.

There is a consensus concerning certain qualitative characteristics of nervous tissue mechanics. For example, it is now widely accepted that this tissue is significantly softer than other tissue types in our body (Discher et al., 2005). Furthermore, nervous tissue shows stress relaxation, its shear modulus decreases to only few hundred Pa within seconds (own unpublished data; Bilston et al., 1997; Georges et al., 2006). Brain tissue is accepted to be strongly inhomogeneous and anisotropic, its mechanical properties regionally differ significantly (Elkin et al., 2007; Gefen and Margulies, 2004). Thereby, the extent of the regional anisotropy strongly correlates with the degree of alignment in the local neuroarchitecture (Prange et al., 2000). Consequently, gray matter exhibits the least anisotropy, while the corpus callosum shows a high degree of anisotropy (Prange and Margulies, 2002). As one further example, it has been shown that the brainstem exhibits an anisotropic response to deformation and that it is stiffer than cerebral tissue (Arbogast and Margulies, 1998).

The important question about postmortem changes of mechanical properties of the delicate nervous tissue, and with it the question about the value of *in vitro* measurements, has been addressed by several authors. It has been suggested

that, since alterations of neurofilaments at room temperature can be detected not before six hours after death (Fountoulakis et al., 2001) and since mechanical material properties follow the material's composition and its structure (Fung, 1993), first changes in the mechanical properties of nervous tissue should not be expected before six hours after dead (Gefen and Margulies, 2004). However, it has been found that the tissue's stress response does show a clear dependency on the postmortem time, becoming stiffer with increasing time (Garo et al., 2007). Interestingly, perfusion does not seem to affect the stiffness of living cortical tissue (Gefen and Margulies, 2004).

Further findings about the mechanics of the CNS include the fact that porcine brain tissue is considerably softer in extension than in compression (Miller and Chinzei, 2002). In contrast to cartilage or other highly hydrated connective tissues, which become stiffer with age (Charlebois et al., 2004), the mature brain appears to be softer than the immature (Gefen et al., 2003; Prange and Margulies, 2002). However, there is again contradictory data that shows nervous tissue to become more resistant to tensile stress with age (Reichenbach et al., 1991a) and to be stiffer in the adult (Thibault and Margulies, 1998).

The mechanical characteristics of biological tissue depends on the passive and active mechanical properties of its constituents (Van Essen, 1997). An investigation of the response of neurons and neuron-like cells cultured in 2D or 3D environments to globally applied shear strains or stresses revealed changes in cellular elasticity, in their cytoskeletal composition, and in their survival rate. The cells' deformation response strongly depends on the severity of the deformation (LaPlaca et al., 1997). The resistance of cells to an applied cyclic shear stress decreases with time, probably as a consequence of the depolymerization of the cytoskeleton (Edwards et al., 2001). Concomitantly, the free intracellular calcium concentration raises with increasing strain (LaPlaca et al., 1997). A further consequence of cyclic shear stress is an increased injury of the cells if compared to the application of steady shear stress (Edwards et al., 2001). Significant cell death occurs following moderate and high rate deformations but not after quasi-static loading (Cullen et al., 2007b). At the same loading parameters, the death rate of neurons cultured in 3D is higher if compared to 2D cultures, probably due to differences in the cellular strain manifestation (Cullen and LaPlaca, 2006). Thereby, the damage depends on the orientation of the neurons within the matrix (LaPlaca et al., 2005).

Consequences of similar mechanical stimuli for glial cells in culture include astrocyte hypertrophy and an overexpression of glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs) (Cullen et al., 2007b), which is similarly observed in gliotic reactions. Also cell death could be demonstrated as a consequence of high rate, high magnitude shear strain for astrocytes (LaPlaca et al., 2005). Again, glial cell death and gliotic profiles depend on the strain rate (Cullen et al., 2007b). Interestingly, in co-cultures of neurons and astrocytes neuronal cell death is highly dominant, suggesting that neurons are significantly more susceptible to mechanical stimuli (Cullen et al., 2007b).

It could furthermore be shown that a subset of genes that are involved in cell death and survival are differentially regulated after dynamically stretching the cells and that the expression of specific genes is correlated with certain parameters of this mechanical stimulation (Morrison et al., 2000). Particularly axons seem to be susceptible to mechanical injury, with rapid stretching being one of the most serious reasons for cell damage. This susceptibility, which is characterized by damage of the axonal cytoskeleton, consequential impairment of axoplasmic transport, axonal swelling, and calcium entry into the damaged axons, is attributed to both their highly organized structure and their mechanical properties (Smith and Meaney, 2000).

However, the ability of neural cells to respond to mechanical stimuli is not limited to externally applied stains or stresses. In fact, the cells are also able to actively probe the mechanics of their environment and to respond to it (Bischofs and Schwarz, 2003; Discher et al., 2005; Franze et al., 2008; Georges and Janmey, 2005; Janmey and Weitz, 2004; Pelham and Wang, 1997). An immense progress in biological sciences was made with the introduction of cell culture systems. The possibility to keep cells alive *ex vivo* allowed the extensive investigation of cellular parameters that would not have been possible *in vivo*. Of course, these artificial cell culture systems differ considerably from the cells' natural environment. The recognition of this circumstance has led to the establishment of cell culture media that regulate the pH and that offer all kinds of nutrition and growth factors to the cells; the main focus has been the maintenance of biochemical parameters. However, it is becoming increasingly clear that also the mechanics of the environment is a crucial parameter for the development and the behavior of cells (Bard and Hay, 1975; Discher et al., 2005; Engler et al., 2004a; Georges and Janmey, 2005; Hay, 1982; Pelham and Wang, 1997).

A key finding, which will certainly influence many future *in vitro* experiments, is that most cells grow differently on substrates with different compliance (Balgude et al., 2001; Engler et al., 2004b; Flanagan et al., 2002; Wang et al., 2000; Yeung et al., 2005). Depending on substrate compliance and cell type, the expression and arrangement of cytoskeletal and membrane proteins including adhesion proteins will change, which is combined with a change in focal adhesions, cell motility and lamellipodial activity, and thus with a change of the whole cell shape and behavior (Discher et al., 2005; Lo et al., 2000; Pelham and Wang, 1997) (Fig. 10.12). If the cells recognize the substrate as "stiff", they will mostly assume a phenotype that is very similar to the one known from classical tissue plastic cultures. For example, glial cells, fibroblasts, or endothelial cells will spread very well on these stiff surfaces, their cytoskeleton will be well arranged, and most cells will develop actin stress fibers. In contrast, if the cells experience the substrate as "soft", they will mostly be rounded up, the organization of their cytoskeleton will be rather diffuse, and cell adhesion will be decreased. Interestingly, the intercellular contact in confluent cultures often overrides the mechanical signaling from the substrate and cells will possess indistinguishable morphologies on soft and stiff substrates

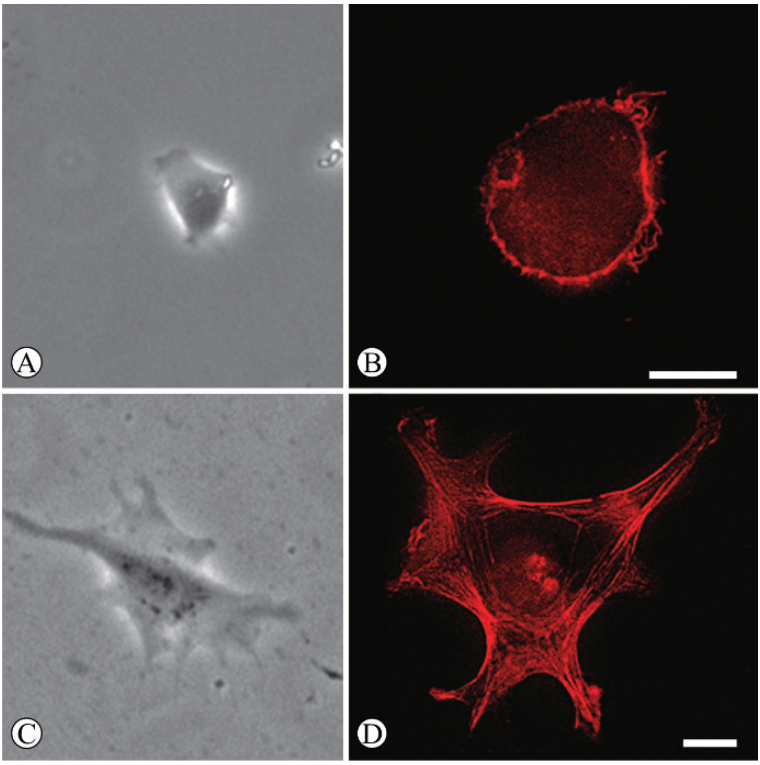

Fig. 10.12 Behavior of cells on deformable substrates.
Fibroblasts on soft gels are rounded up (A), and their actin cytoskeleton appears rather homogeneous (B). In contrast, if the cells are cultured on stiff gels, they are flatter and extend processes (C); their actin cytoskeleton shows stress fibers, which resemble those known from normal tissue cultures on glass or plastic. Adapted from Georges et al., 2005

(Yeung et al., 2005). In both cases they assume a rather "stiff phenotype": Independently on the substrate compliance they appear well spread and rather flat.

There is a large divergence in the response of different cell types to a substrate with a specific compliance. Cells seem to possess a certain "tactile set-point", similar to biochemical set-points such as ion concentrations (Discher et al., 2005). Consequently, most tissue cell types have a preferred range of substrate elasticities, and they will behave differently in soft and stiff environments. The transition between "soft and stiff phenotypes" mostly seems to occur over a fairly narrow range of substrate compliance. While changes in morphology and also in motility of fibroblasts occur at substrate stiffnesses of about 14–30 kPa (Lo et al., 2000), neurons change their growth behavior at substrate stiffnesses of about two orders of magnitude below these values. Neurons have been observed to significantly increase the length of their neurites and the number of

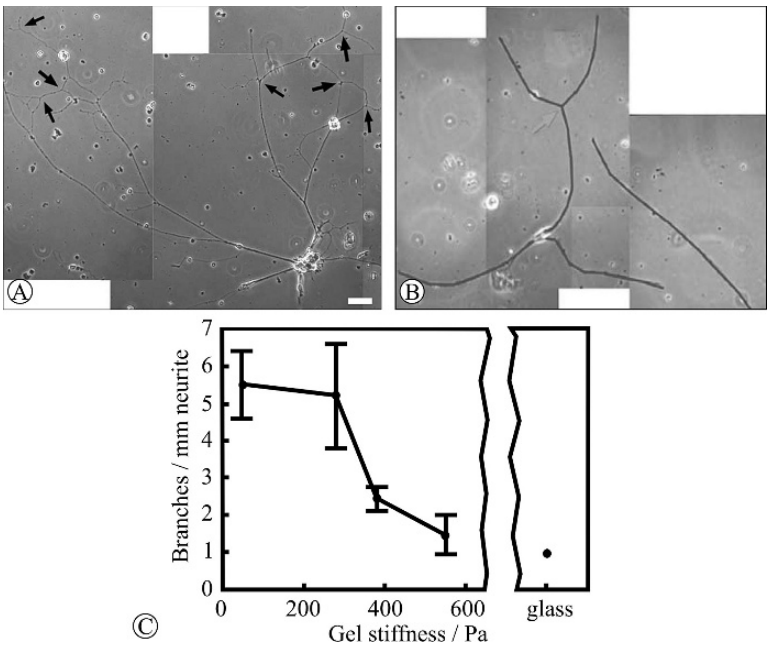

Fig. 10.13 Neuronal growth in dependence on the substrate compliance.
(A) If neurons are cultured on substrates they recognize as "soft" they extend neurites with multiple branch points (marked by arrows). However, if neurons grow on substrates they identify as "stiff" the number of branch points significantly decreases (B). (C) Quantification of neurite branching in dependence of substrate compliance. A relatively sharp transition from neurons with many branches to those with significantly less branches occurs at substrate elasticities of about 300 Pa. Scale bar in (A): 50 μm, also applies for (B). Adapted from Flanagan et al., 2002

side branches on soft substrates (Balgude et al., 2001; Flanagan et al., 2002). Flanagan and colleagues have found a rather sharp transition from neurons with many branches to those with significantly less branches at a substrate stiffness of about 300 Pa (Flanagan et al., 2002) (Fig. 10.13). In three-dimensional cultures, increasing gel stiffness is furthermore accompanied by a decrease in the rate of neurite extension, eventually due to the increase in polymer density (Balgude et al., 2001).

Thus, the experience of substrate deformability is strongly cell type specific. While fibroblasts could hardly mechanically distinguish substrates in the neurons' critical range, the substrates fibroblasts are sensible to would likely appear infinitely stiff to neurons. The branching frequency of neurons on gels of 550 Pa has been observed to be very similar to the branching frequency of neurons cultured on glass, whose elastic modulus is in the order of tens of GPa (Flanagan et al., 2002) (Fig. 10.13).

Mixed cultures of neurons and glial cells also assume a characteristic, substrate compliance-dependent appearance. On gels of few hundred Pa ("soft"),

astrocytes are small and round and neurons extend long neurites. The density of astrocytes decreases with time. On substrates of few kPa ("stiff"), astrocytes spread and adhere considerably better, their projected area is significantly larger than on soft gels (Georges et al., 2006). It is worth mentioning that astrocytes cultured on these substrates assume a phenotype resembling reactive cultured glia. Interestingly, in the presence of glial cells, neuronal morphology and adhesion on stiffer substrates is very similar to that on soft ones. However, on stiff substrates neurons grow almost exclusively on top of glial cells, which most likely offer the compliance that is needed by neurons (Lu et al., 2006). Moreover, neurons seem to be able to grow independently of astrocytes only on soft substrates. The ratio of neurons to astrocytes decreases drastically with increasing substrate stiffness (Georges et al., 2006). Finally, F-actin in astrocytes is mainly disordered if cultured on soft substrates. Abundant stress fibers are only found on hard surfaces. Neurons by contrast are able to polymerize F-actin independent on substrate compliance. However, on soft gels the F-actin content of neurons exceeds that on stiff gels by 30% (Georges et al., 2006).

The way how cells sense the mechanics of their environment is very similar to the way we would judge the compliance of an object. Most tissue cells or at least parts of them anchor and continuously exert forces on their substrate, thereby deforming it (Bray, 1979; Griffin et al., 2004b; Harris et al., 1981; Harris et al., 1980; Pelham and Wang, 1997). This contractility does probably not arise from a universal requirement of cells to exert forces on their surrounding. Rather, these traction forces are likely a result of an active, regulated response to the mechanical resistance that the cells feel. The cells probe the mechanics of their substrate and respond to it by adjusting adhesion and cytoskeleton, and as a result the net contractile forces and their overall state (Discher et al., 2005). In contrast to astrocytes, neurons have been found to exert comparatively very little stress to their surrounding (Bridgman et al., 2001; Franze et al., 2008; Harris et al., 1981). This could explain why neurons can not mechanically distinguish gels as soft as 550 Pa from glass (Flanagan et al., 2002).

Interestingly, if neurons embedded in 3D cultures are exposed to high-rate deformations, their viability significantly decreases with increasing substrate stiffness (Cullen et al., 2007a). Ultimately, the substrate compliance alone is sufficient to determine the fate of stem cells. If naive mesenchymal stem cells are seeded on matrices with a compliance comparable to that of soft brain tissue, they will likely give rise to neurons (Engler et al., 2006). In conclusion, the data currently available point to a strong preference of neurons for very soft environments.

The importance of mechanical cues for the neuronal development has furthermore been demonstrated in a different context. First pioneering work has shown that neuronal growth cones, which are the structures responsible for the recognition of guidance cues, can exert mechanical tension on their neurites (Bray, 1979; Joshi et al., 1985). The tension on a neurite has been determined

to be on the order of 30–40 μdyn (300–400 pN) (Dennerll et al., 1988). The application of this tension by the growth cone is possible due to its comparatively strong adhesion to the substrate, which increases with contact area. The forces required to detach growth cones *in vitro* have been measured to be between 760 and 3340 μdyn, and exceed thus the typical resting axonal tension and the force exerted by advancing growth cones (Lamoureux et al., 1989) by roughly an order of magnitude (Zheng et al., 1994). Tension also seems to be the driving force of neurite retraction, which occurs *in vitro* (Bray, 1979; Dennerll et al., 1989; Joshi et al., 1985) as well as *in vivo* (Riley, 1981). The cytoskeleton is a key player in its maintenance: While actin supports (or causes) the neurite tension, microtubules work against it (Dennerll et al., 1988; Dennerll et al., 1989).

PC12 neurite spring constants have been calculated to be about 24 μdyn/μm (240 μN/m) (Dennerll et al., 1988), neurites of dorsal root ganglion cells are apparently stiffer (Dennerll et al., 1989); forebrain neurons in turn show only weak elastic behavior (Chada et al., 1997). The application of tensions to neurites of PC12 cells and peripheral neurons below about 100 μdyn caused a passive response and revealed their viscoelastic, solid nature. However, if these externally generated tensions exceeded the threshold of 100 μdyn, neurite outgrowth could be induced (Dennerll et al., 1989; Zheng et al., 1991). This process has been termed "towed growth" (Bray, 1984; Heidemann and Buxbaum, 1994) (Fig. 10.14). In contrast, for the elongation of neurons of the CNS no minimum threshold tension is required. Here, all positive tensions stimulate neurite outgrowth (Chada et al., 1997). Hippocampal neurites, which result from towed growth, assume axon-like characteristics, suggesting that tension is an important determinant in axonal specification (Lamoureux et al., 2002). The elongation rate of neurites from both peripheral and central nervous system due to towed growth increases proportionally to the tension magnitude; an increase

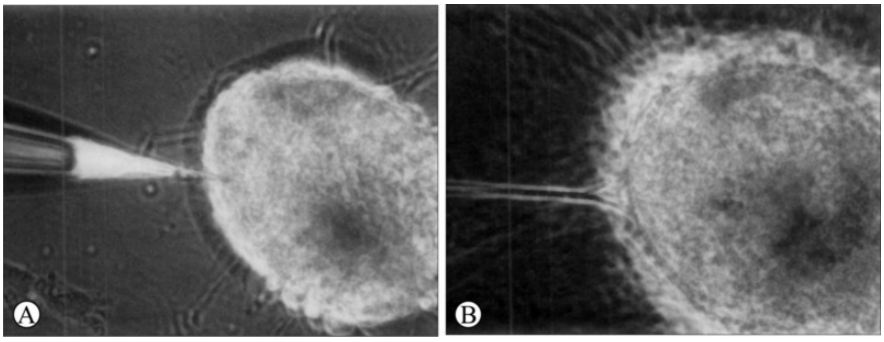

Fig. 10.14 Towed growth of a neurite.
If tension is applied to an existing neurite or to a neuronal cell body (A), a neurite can either be elongated significantly or even be generated de novo (B), respectively. Magnification ×132. Adapted from Bray, 1984

in tension of 1 μdyn results in an increase of the elongation rate of about 1–1.5 μm/h (Chada et al., 1997; Zheng et al., 1991). Thus, processes of neurons of the CNS show a significantly less elastic behavior than those of sensory neurons, which behave as passive viscoelastic materials after mechanical stimulation below threshold, and which actively respond to forces that exceed a threshold (Franze et al., 2008).

In conclusion, similar to cells and cell parts that constantly pull on their environment growth cones additionally pull on their neurites. In both cases the passive and active mechanical properties of the cells allow them to integrate external mechanical signals. A feedback mechanism then tends to maintain steady tension, thus triggering cell responses which mainly concern the organization of the cytoskeleton and of adhesions in case of cells in compliant environments and the adjustment of neurite length in case of the developing neurite (Van Essen, 1997).

These data suggest that the mechanical properties of the neuronal environment *in situ* are of immense importance for the development, normal functioning, and eventually also for the pathology of the nervous system. *In vivo,* this environment mainly consists of glial cells, which fill most of the space between the neuronal elements, other neurons, and extracellular matrix. Current assumptions about glial cell mechanics were contradictory. As already mentioned, glial cells are thought to glue the neurons together (Virchow, 1856). However, they are also assumed to provide a rather stiff structural scaffold to mechanically support neurons like a wall supports ivy (e.g., the term "support cells" is used for glial cells of sensory organs such as the retina) (Schultze, 1866). While glue at the times of Virchow was rather soft and viscous (i.e., non-elastic, like honey), mechanical support cells should be stiff and dominantly elastic, like the piers of a bridge. Thus, both of these two widely spread assumptions – assuming either a more fluid or solid character of glial cells – are mutually exclusive. Only recently, we have quantified viscoelastic properties of acutely isolated neurons and glial cells of two different CNS regions, and we could disprove both of these hypotheses.

Our study was conducted on cells of the hippocampus, which is a part of the brain, and on retinal cells. Using scanning force microscopy (SFM) (Mahaffy et al., 2004; Mahaffy et al., 2000), we have measured local frequency dependent complex Young's moduli $E^* = E' + iE''$ of the principal glial cells of the respective tissues (astrocytes in the hippocampus and Müller glial cells in the retina) and of their neighboring neurons (pyramidal cells and retinal interneurons, respectively) (Lu et al., 2006).

All CNS cells investigated behaved dominantly elastic ($E' > E''$), which disproved the idea of glial cells acting as viscous putty or glue. Furthermore, in both CNS regions glial cells were about twice as soft as their neighboring neurons. Therefore, they can not offer any mechanical support to neurons. The dominating elastic storage modulus E' of the somata of pyramidal neurons is in the range of 500–1000 Pa at frequencies between 30 and 200 Hz, while that of their surrounding astrocytes amounts only to about 300–500 Pa in the same

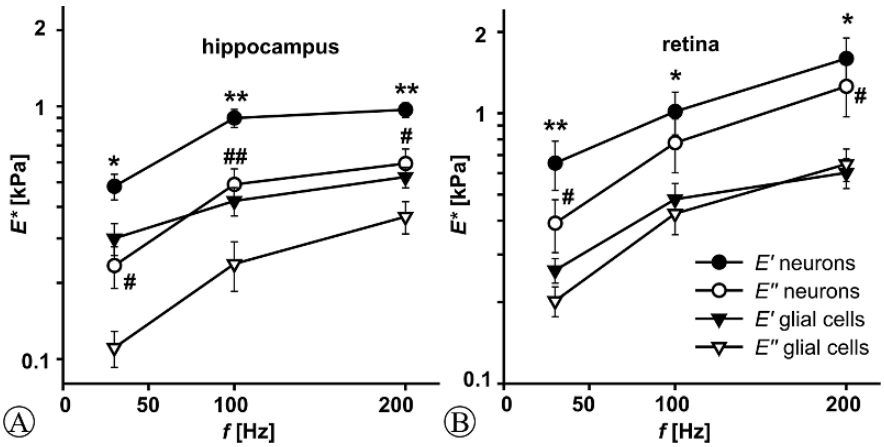

Fig. 10.15 Viscoelastic properties of neurons and glial cells in the CNS.
In the hippocampus (**A**) as well as in the retina (**B**) all cells behave dominantly elastic (since the storage modulus E' significantly exceeds the loss modulus E″). Furthermore, in both tissues glial cells are significantly softer than their neighboring neurons (since their storage modulus is smaller). Adapted from Lu et al., 2006

frequency range. In the retina, E' of the neuronal somata assumes values between about 650 and 1600 Pa, whereas the adjacent Müller cell somata show storage moduli in the range of about 250–600 Pa at frequencies between 30 and 200 Hz (Fig. 10.15).

The greater stiffness of neurons leads to a compression of their neighboring, softer glial cells, as has been confirmed using electron microscopy (Lu et al., 2006) (Fig. 10.16). However, even neurons are comparatively soft. Fibroblasts

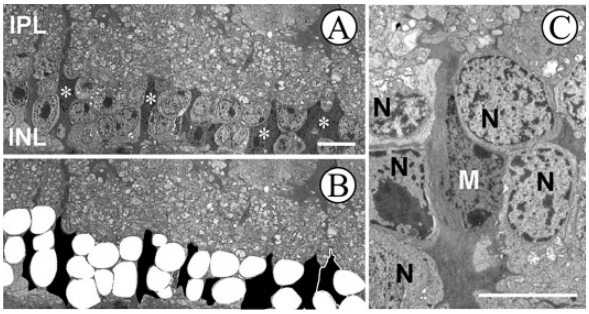

Fig. 10.16 Transmission electron micrographs of the inner nuclear layer of a retina.
(A) Glial cell somata, which are marked with asterisks, appear darker, because their cytoplasm is electron-denser than that of the retinal neurons (Germer et al., 1998). (B) Same image; glial cell somata are labeled in *black*, neuronal somata in white. The somata of (the stiffer) bipolar cells are smoothly rounded and indent the neighboring, irregular-shaped (softer) Müller cell somata. (C) The same image at higher magnification. Scale bars: 10 μm. Adapted from Lu et al., 2006

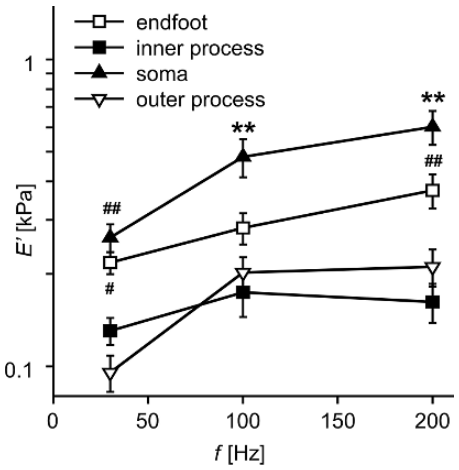

Fig. 10.17 Spatial elasticity distribution along a retinal glial cell. Despite containing densely packed components of the cytoskeleton, both cell processes are significantly softer than the cell soma and the funnel-shaped termination of the inner process ("endfoot"). While the elasticity of these processes barely reaches 200 Pa, the elasticity of most other cellular components found in the retina exceeds 300 Pa. Adapted from Lu et al., 2006

for example are about twice as stiff as the stiffest cellular structures we found in the CNS (Mahaffy et al., 2000).

Similar as the cells' cytoskeletal distribution also their mechanical properties vary in different regions. Despite both stem processes of the radial retinal glial cells are densely packed with cytoskeletal elements (Reichenbach et al., 1988a; Reichenbach et al., 1988b), they are significantly softer than any other cellular structure in the tissue. Their elastic moduli are in the range of about 100–200 Pa at 30–200 Hz. In contrast, the cell soma, which contains the nucleus, has a storage modulus of ~250–600 Pa, and the endfoot, which is a funnel shaped termination of the inner process that contains mainly smooth endoplasmatic reticulum, assumes intermediate values between $E' \approx 200$–400 Pa in the same frequency range (Fig. 10.17). Similarly, also the processes of neurons are significantly softer than their somata (Lu et al., 2006).

Finally, a qualitative analysis of the response of glial cells to externally applied mechanical stresses validated their elastic nature. The deformation of whole glial cells with an *optical stretcher* (Guck et al., 2001) revealed that they mechanically behave similar to shock absorbers. These results suggest that glia in the CNS may be comparable to cushioning material in common packaging. Thus, they might form a protective soft, compliant matrix around the neurons and minimize damage in cases of mechanical trauma (Lu et al., 2006).

10.6 Viscoelastic Properties of CNS Cells: Impact on Development, Physiology, and Pathology

All biological objects follow physical laws. Mechanics is crucial for passive cellular tasks, e.g., the maintenance of their structure, as well as for their active behavior, e.g., interactions with their environment. The investigation of

passive mechanical properties and active mechanical behavior of biological cells and tissues is therefore indispensable for a comprehensive understanding of live.

10.6.1 Cellular Biomechanics in CNS Development

Cellular biomechanics has been suggested – or even shown – to be crucially involved in several important events of CNS development. First, the massive progenitor cell replication in the early neural plate not only drives its enlargement but also appears to be involved in its re-shaping into a neural tube. Cell division always occurs at the apical surface of the neuroepithelium (the surface originally facing the outer surface of the embryo, later the lining of the ventricular system). Then, by a process called interkinetic migration, the somata of the progenitor cells move towards the opposite surface where the S-phase of their mitotic cycle takes place. Finally, the somata move back to the ventricular surface, and quickly divide. Because the duration of S-phase is much longer than that of the subsequent phases, most of the cell nuclei with duplicated DNA content (i.e., with bigger size) are close to the basal surface while the small nuclei with single DNA content are close to the apical surface. This unequal distribution has been suggested to contribute to the enrolling of the neural plate, with the originally outer surface now facing the inner surface of the tube (Jacobson, 1991).

Later in ontogenetic development, the unequal time courses of growth and maturation of the CNS and its "exoskeleton" significantly contribute to the shaping of the nervous tissue. For instance, at early stages the future skull is assumed to be softer than the growing brain, and thus the bulging hemispheres drive the enlargement of the skull. The stiffness of the skull increases with time and the expansion of the brain becomes limited. As the neuroepithelial cells continue dividing, the surface of the cortex further enlarges. The postmitotic newborn neurons migrate from the ventricular zone towards the cortical plate according to an inside-out sequence. Each new wave of neuroblasts migrates through the previous cell generation to occupy a more superficial position. Consequently, the outer cortical layers possess a greater surface area than the inner layers, which is thought to provoke a compressive force that is involved in the formation of sulci and gyri (Couillard-Despres et al., 2001). By contrast, the growth of the spinal column is prolonged in comparison to that of the spinal cord. Thus, the nerves must be stretched or elongated in order to maintain the connection to their targets and to follow their initial paths through the "exits" between the vertebrae. This process, which likely involves mechanical stretching of the nerves and which highly resembles "towed growth" observed *in vitro* (Bray, 1984), leads to the formation of the so-called *cauda equina*. This observation suggests tension to be an additional cue for neurite growth and thus a crucial factor in CNS development (Lamoureux et al., 2002; Van Essen, 1997).

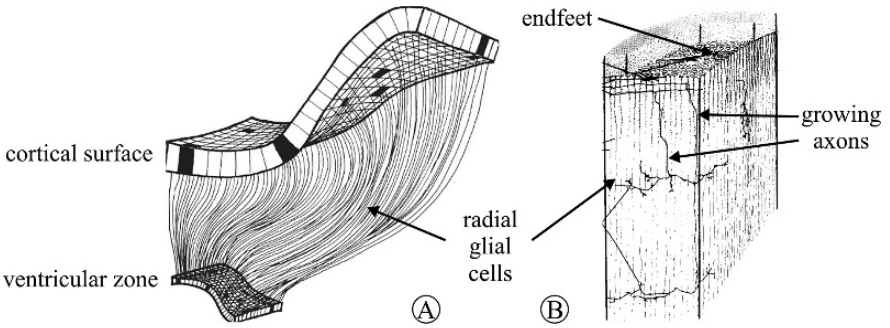

Fig. 10.18 Migration and growth patterns of developing neurons.
(**A**) In the developing cortex, neurons migrate radially outward from the ventricular zone, the site of their origin, to the cortical surface. Thereby, they use radial glial cells, which connect both cortical surfaces, as substrate for their migration. Neurons strictly follow these cells, even when the radial glial cells are significantly bent (adapted from Rakic, 1972). (**B**) Axons growing *in situ* along superficial tissue layers may turn right-angled and penetrate deeper layers. Thereby, their growth cones follow again the soft radial glial cell processes (adapted from Vanselow et al., 1989). In both cases, the softness of radial glial cells could be used as guidance cue that conducts neurons to their final destination

Throughout neuronal migration and growth, the scaffold of radial glial cells, which connects the ventricular with the cortical surface, may provide important guidance cues. During cortical development, neurons migrate along radial glial cell processes (Rakic, 1972). Furthermore, axons growing *in situ* along superficial tissue layers turn right-angled and penetrate deeper layers (Vanselow et al., 1989). Thereby, their growth cones follow again the radial glial cell processes (Fig. 10.18). Migrating cells have been shown to use the compliance of their environment as guidance cue, a process called mechanotaxis (Lo et al., 2000). As mentioned in Section 10.5, glial cells are softer than neurons (which prefer soft substrates). Thus, they may provide mechanical cues for neuronal migration and neurite growth, both processes which are absolutely essential for the proper development of the nervous system. Intriguingly, the storage modulus of radial glial cell processes in the adult retina does not exceed 200 Pa (Lu et al., 2006), which is below the value at which neurons *in vitro* change their growth behavior (Flanagan et al., 2002). If radial glial cell processes in the developing CNS are similarly soft, migrating neurons and growing neurites could use the glial cells as soft mechanical guideposts – in a complex interplay with other cues.

Finally, it should be pointed out that developmental biomechanics is not only important for the brain and the spinal cord but also for the retina as an ontogenetically evolved part of the CNS. For example, the so-called balloon model of eye growth explains the postnatal increase in retinal surface area (which occurs *after* all cells have been generated) by a passive, indirect process. In this model, the intraocular pressure acts on the “exoskeleton” of the eye, the

sclera, and drives its enlargement (Mastronarde et al., 1984). The retina, which is only fixed to the eye at its anterior, circular margin, is then passively stretched like a glove when the fist enters it. This passive growth of the retina causes its areal expansion at the expense of thinning of the tissue and a disproportional expansion. The disproportional expansion can be explained by the different biomechanical properties of local retinal tissue. The retinal center is stiffer than the periphery (Reichenbach et al., 1991a) and becomes thus less stretched by the expanding "balloon". Thus, the cellular density and therefore the visual acuity are higher in the central than in the peripheral retina. During ongoing stretching, this difference is maintained or even enhanced, and shapes the retinal center as visual streak or area centralis (Reichenbach et al., 1991b). In an even more complicated process, mechanical tension is thought to be a main contributor to the formation of the fovea during the late development of the primate and human retina (Provis et al., 1998; Van Essen, 1997). It remains to be elucidated how these complex biomechanical events contribute to the pattern formation of retinal cells (and cell populations) and to the shaping of the dendritic and receptive fields of retinal neurons (Deich et al., 1994; Reichenbach et al., 1991a; Reichenbach et al., 1993).

10.6.2 Cellular Biomechanics in CNS Functioning, Plasticity, and Pathology

Under physiological conditions, the passive mechanical material properties of the CNS' principal building stones, neurons and glial cells, separate these cell types in two distinguishable populations. There seems to be a dependency between the material properties of the cells and the mechanical needs of their neighbors. The stiffer neurons prefer soft substrates, while the softer glial cells prefer stiffer ones (Georges et al., 2006; Lu et al., 2006). Hence, neurons and glial cells complement one another not only in terms of biochemical interactions but also by providing the mechanical substrate the partner requires. The glial cells may thus provide a soft, compliant embedding for the neurons, which can even accommodate the swelling of neuronal compartments during neuronal activity (Pannicke et al., 2004; Uckermann et al., 2004). In this context, it is interesting to note that in the retina all synapses and most neuronal processes are located within the plexiform layers, where glial cells are softest (Lu et al., 2006) (Fig. 10.19). Furthermore, extensive growth of neuronal processes occurs in the retina after cellular degeneration (Lewis et al., 2003; Peichl and Bolz, 1984). The sites of such growth are exactly those plexiform layers, which suggests that developing neuronal processes may be attracted by soft environments – or repelled by stiff ones.

On the other hand, also in certain pathological events in the CNS biomechanics can be important. The retina for example is held in place by a fine balance between the intraocular pressure and negative pressure from the site of

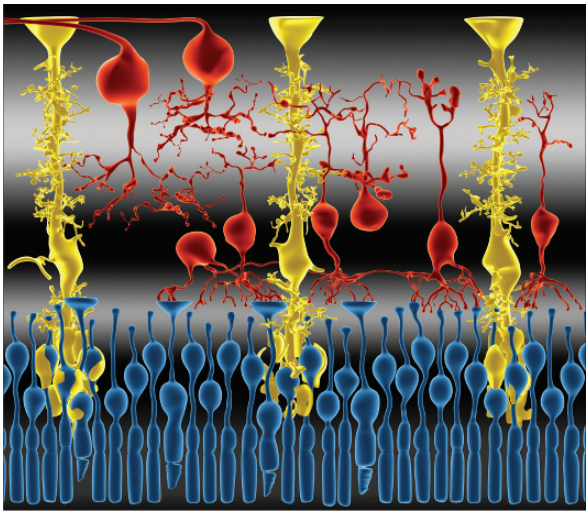

Fig. 10.19 Schematic of the stiffness gradient in a retina. The spatial differences in glial cell stiffness are indicated in the black and white overlay. Interestingly, most branches of neuronal processes and all synapses are found in those retinal layers where the glial cells are softest. Adapted from Lu et al., 2006

the retinal pigment epithelium (Yao et al., 1994). Several retinal disorders such as retinal detachment, retinoschisis, and proliferative vitreoretinopathy are thought to be caused by mechanical stresses and may be (Reid et al., 2003) or may not be (Mooy et al., 2002) facilitated or even caused by anomalous mechanical properties of retinal (glial) cells.

In cases of rapid mechanical trauma such as in car accidents, the hard "exoskeleton" of the CNS may become detrimental. Because of inertia, the nervous tissue is pushed against skull, vertebrae, or sclera, and may thus be deformed and damaged. The inhomogeneous, anisotropic structure of the brain leads to a characteristic stress distribution within the tissue (LaPlaca et al., 2007). This stress distribution causes regionally different degrees of cell damage, depending on the tissues' local complex viscoelastic behavior. Important contributions to this behavior include the mechanical properties of the cells, of the structures that bind them together, such as the extracellular matrix and intercellular adhesion molecules, and (inter)cellular forces (Moore et al., 1995). Therefore, extended studies of the response of central nervous tissue and its constituents to mechanical stresses will help to understand the mechanisms behind traumatic neuronal damage.

In any case of neurotrauma, neuronal damage is accompanied by more or less effective attempts of neuronal regeneration. This regeneration requires the (re-) growth of neurites from the surviving neurons and may be facilitated by soft glial cell processes. However, it is known that glial cells form scar tissue in various cases of neuronal injury or degeneration (Fawcett and Asher, 1999; Logan and Berry, 2002). These scars are thought to be stiffer than neuronal processes. Since neurons prefer soft substrates (Balgude et al., 2001; Flanagan et al., 2002), stiff scars might thus constitute a mechanical (and biochemical)

barrier and impair efficient neuronal regeneration in mammals (Fawcett and Asher, 1999; Horner and Gage, 2000; Yu and Bellamkonda, 2001), but this hypothesis remains to be proven. It is intriguing to speculate whether glial scars in lower vertebrates, where neurons can regenerate, are softer than in mammals.

10.6.3 Outlook

It is evident that *cellular neuromechanics* is a very young, developing field, just in the stage of raising many new questions rather than generating answers. Already now it can be expected that many of these questions will lead to answers which will have great impact for our understanding of the normal and disturbed development and functioning of the nervous system. As one example, the re-growth of axons in the injured CNS may constitute a field which may greatly benefit from biomechanics. Particularly, the obvious preference of neurons for soft substrates might be exploited to stimulate neurite outgrowth and to guide these neurites to their desired destination. The implantation of soft hydrogels into nervous tissue lesions has been shown to have beneficial effects on neuronal repair, providing both neuronal ingrowth as well as the reduction of the glial scar (Lesny et al., 2002; Woerly et al., 2004). These beneficial effects of the gels might at least partly be attributed to their softness. The matching of the mechanical properties of prostheses used for bridging lesion sites in the nervous system to the requirements of the (damaged) neurons might also have desirable effects on the production and release of inflammatory mediators in their neighborhood. Soft implants might furthermore avoid the stimulation of glial cells to become reactive (as observed *in vitro* on hard substrates), thus reducing scar formation. These altered cell responses would likely lead to a decreased foreign body reaction and to an increased biocompatibility of the implant.

In conclusion, the mechanical properties of the cells of the nervous system may be important throughout life, *viz*. for neuronal development and normal adult plasticity as well as for regeneration. Ultimately, *neuromechanics* might thus significantly contribute to overcome the dogma of the impossibility of neuronal repair in the CNS.

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Chapter 11
The Neural Representation of Kinematics and Dynamics in Multiple Brain Regions: The Use of Force Field Reaching Paradigms in the Primate and Rat

Joseph T. Francis

Abstract In this chapter I will review the past and present motor control literature with an emphasis on reaching movements. Debate still rages as to what movement related variables are controlled by the neural motor control system especially concerning dynamic vs. kinematic variables. There is a rich history in the motor control literature that has employed monkeys of several species making reaching movements while holding loaded or robotic manipulandum. However, to date very little work has been conducted using the rat reaching paradigm to investigate the control of dynamics vs. kinematics. I will review a very simple rat robotic manipulandum paradigm and initial results from it. This paradigm is presently being expanded from a 1 degree of freedom system similar to those used in early primate research to a 2 and even 3 degree of freedom version, such as those used with primates and humans today.

Keywords Motor learning · brain machine interface · thalamus · somatotopic · somatosensory prosthesis

11.1 Introduction

Much of the mammalian nervous system has evolved around a common set of basic needs, such as obtaining food and evading predators. What elements are needed to carry out such basic operations by an animal? The animal must be able to sense the food object, or predator, and be able to reach/grasp or move to the food, or flee from the predator. Having several senses would certainly be

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beneficial to the animal as it could use correlations between the senses to learn about its environment and its own self generated movement. Even single celled protozoans, such as paramecium and euglena, have multiple senses such as chemical (taste/smell), Photo (sight), electric field, gravitational and mechanical sensors. Animals also need to be able to move to act on the sensory information, and in general animals learn to make certain sensory predictions based on their own motor commands. When these predictions are incorrect learning should takes place.

How does our nervous system use this sensory information and execute movements, and in what form is the information encoded within the neural substrate? Obviously I will not be able to fully discuss these questions in this chapter; however, I will attempt to review those aspects that stem from our sense of proprioception, as it stems from mechanical receptors, the theme of this book. Specifically, I will focus on the neural representation of dynamics (force related) and kinematics (position related) neuronal activity, or at least neural activity that correlates well with these two groups of variables. In addition, I will be limiting my discussion to primates and the rodent, as well as making comparisons between these two.

11.1.1 Reaching Movements

I am going to use targeted reaching movements as the basic sensory motor task for our discussion. Reaching to grasp an object involves a great deal of neural processing, ranging from the computations that relate external space to our intrinsic motor apparatus, to sensing the size and compliance of target objects. The sensory motor control system involves interaction between feed-forward and feedback signals. The CNS uses visual, proprioceptive and cutaneous information to make natural reaching movements and to facilitate motor learning (Polit and Bizzi 1979; Lackner and Dizio 1994; Ghez et al. 1995; Gordon et al. 1995; Sarlegna et al. 2004; Sober and Sabes 2005). Moreover, the sensory motor control system can switch between these sensory modalities in relation to their reliability and the goals of the task (Sarlegna et al. 2003; Sober and Sabes 2003; Sarlegna et al. 2004; Scheidt et al. 2005; Sober and Sabes 2005). Whereas vision can guide the kinematics of movement, it cannot easily detect the loads that must be overcome. The proprioceptive system can detect loads as well as information about the arms position, velocity and acceleration. However, it has been suggested that the proprioceptive system drifts (Wann and Ibrahim 1992) over time unless it is intermittently reset via visual or tactile input. There is still much debate as to the cause of proprioceptive drift as well as its extent. The ability of visual input to ameliorate such drifting and its overall influence on reaching is apparent in individuals with large fiber neuropathy (Ghez et al. 1995; Gordon et al. 1995). However, these same individuals make gross errors when vision is occluded. It is obvious that both vision and proprioception play vital roles in motor

control. Thus, reaching movements normally depend on visual, proprioceptive as well as tactile information for humans, and primates in general (Lackner and Dizio 1994; Graziano 1999).

For the past several decades neurophysiologists and psychophysicists have worked to elucidate the control mechanisms employed by the brain and spinal cord that allow us and other animals to move voluntarily with a great degree of plasticity. Much of this work has been dedicated to determining which variables of movement we specifically control. In addition, there has been a great deal of debate as to what specific brain regions code for which of these variables. However, many frontoparietal brain regions have been shown to encode movement related parameters such as the target (Andersen et al. 1985) and hand positions, (Tillery et al. 1995; Battaglia-Mayer et al. 2000; Graziano et al. 2000; Buneo et al. 2002; Taylor et al. 2002; Carmena et al. 2003) direction (Georgopoulos et al. 1982), velocity (Moran and Schwartz 1999b) and force (Kalaska et al. 1989; Alexander and Crutcher 1990b; Crutcher and Alexander 1990; Li et al. 2001; Scott et al. 2001; Xiao et al. 2006). A more correct statement would be that correlations have been found between the aforementioned variables and neural activity. To date there is still heated debate as to whether the motor cortex and other motor related regions are encoding kinematic, or dynamic related variables, as well as what coordinate system these regions use, such as extrinsic vs. intrinsic coordinate systems. Similar types of questions have been raised regarding information carried in the spinal cord from peripheral mechanoreceptors such as muscle spindles and joint receptors (Bosco and Poppele 2001; Stein et al. 2004).

For rodents, such as the rat and mouse, making reaching movements involve a somewhat different situation than that for primates, as it has been suggested they use olfaction (Whishaw and Tomie 1989) to locate the target object and most likely can't see their forepaws within a large region of the forepaw range of motion. In addition to olfaction, they do have the sense of proprioception, and whiskers, both on their snout as well as whisker like guard hairs on their wrists. Traditionally researchers have used human or non-human primates for the study of targeted reaching movements; however, with the ability to genetically manipulate mice, the low cost associated with rodent work, and the large number of animal models based on the rat and mouse there has been a surge in the use of rodent reaching paradigms. Our understanding of the rodent motor control system is still lagging that of primates, for instance, we have only recently identified the major proprioceptive relay nucleus in the rat thalamus, something that has been long known and studied in the primate (Francis et al. 2008). Our knowledge on the rat's visual system is also lagging as far as the functional neurophysiology. There is a good deal of evidence that primates make their reaching plan in a visual coordinate system, but what about the rat? Do rats plan reaches in a whisker, olfactory, visual, or some other coordinate system? This is an open question which we hope to address in the near future.

11.1.2 The Robotic Manipulandum and the Force Field Paradigm

During a natural reaching movement there is often a large degree of correlation between several movement related variables. If our goal is to determine what movement related variables the different brain regions encode we need to decouple these inherent correlations, and it is for this reason that the loaded and robotic manipulandum have been developed.

Center out reaching paradigm: In the lower half of Fig. 11.1 is a cartoon of an individual holding onto one type of endpoint robotic manipulandum (bottom) that moves in the horizontal plane. In this widely used paradigm subjects are instructed to make a reaching movement to one of the eight peripheral targets seen at the top of the figure, starting all reaches from the center target. This is the standard center out reaching paradigm. Only one reaching target is cued at a time and may require the subject to wait before a go cue is given to actually make the overt reaching movement. The targets may be projected onto the same plane that the subject is reaching in, or can be represented on a computer monitor placed directly in front of the subject with a visual feedback computer cursor representing the position of the hand. This hand position is read from joint encoders on the manipulandum.

By using robotic systems we can ask questions about both kinimatic and dynamic related variables while changing the sensory motor transformations necessary for the subject to complete the task. For instance, one can have the manipulandum produce a velocity dependant curl field, which produces forces tangential to the direction of motion and proportional to the speed, and is complex enough that the subject does not explicitly know what has changed

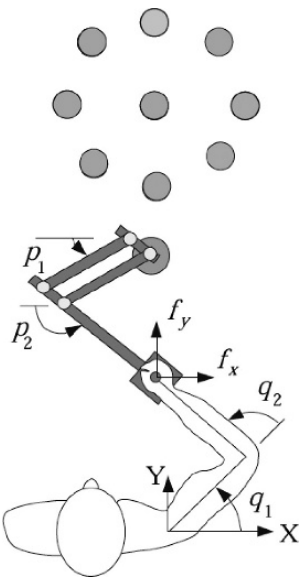

Fig. 11.1 A cartoon of the center our reaching paradigm. A subject, *bottom*, holds onto a robotic manipulandum, *top*. Shown are the joint angles for the subject and manipulandum and forces at the handle

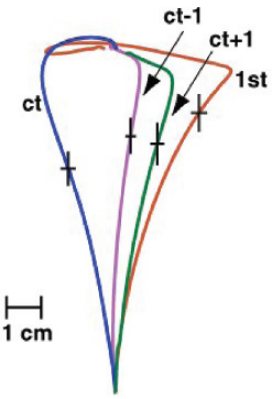

Fig. 11.2 Plotted are the mean reaching trajectories for a group of subjects during the curl field paradigm. During the first fielded movement (1st) the subjects' movements are severely deviated to the right. After learning (ct-1) the movements become straighter indicating the subjects have learned to compensate for the force field. During certain movements the force field was turned off (ct) causing a deviation to the left, that is an error in reaching. This error causes learning to occur, which can be tracked on a trial-by-trial basis, as seen by the difference between ct-1 and ct + 1. (Taken from (Thoroughman and Shadmehr 2000) with permission)

(Shadmehr and Mussa-Ivaldi 1994). When first exposed to such a field subject's movements are severely displaced as seen in Fig. 11.2 labeled as 1st. However, after learning (labeled ct-1) the subjects' trajectories become fairly straight as those seen before the force field was turned on. Thus, we now have a situation where the kinimatics of the movements are similar, but the forces needed to make the movement are very different. We can also secretly turn the force field off, called a catch trial (ct), as the electric motors of such robotic systems produce no discernable noise for the subjects to use as cues. These ct induce large movement errors and can be used to probe how the sensory motor control system learns on a trial-to-trial basis (Thoroughman and Shadmehr 2000; Donchin et al. 2003; Francis 2007). These robotic systems can also be used to change the mapping between the subjects hand and the visual feedback cursor, as well as combining force and these visual perturbations. In Section 11.3 of this chapter we discus results from several neurophysiological studies using the robotic manipulandum.

11.2 Proprioception

11.2.1 Peripheral Mechanoreceptors

Proprioception is defined as the sense of joint position while Kinesthesia is the sense of joint motion. However, often these terms are intermingled and for the

purpose of this chapter I will use the term proprioception to indicate the sense of position and motion of ones body derived from mechanosensitive structures. In the skin there are six types of mechanoreceptors, Meissner's corpuscles, Merkel's endings and nerve endings around hair follicles, all of which are predominantly involved in the sense of touch. In addition, Ruffini endings sense pressure while Pacinian corpuscles sense vibration. There are also free nerve endings that sense pain, temperature and touch, for review see (Nolte 2002). It has been suggested that the skin with these receptors can add to the sense of proprioception (Moberg 1983) when the skin is deformed due to our own movement.

Although the skin may take part in proprioception, the majority of this information comes from two types of mechanoreceptors, **Golgi tendon organs (GTOs)** and **muscle spindles** (for great interactive models of both these receptor types please see Arthur Prochazka's web page, www.ualberta.ca/~aprochaz/research_interactive_receptor_model.html). Golgi tendon organs are located at the connection between muscles and their tendons. They are comprised of collagen surrounded by a sheath called a capsule. Inserted into the collagen matrix are the arborized endings of a 1b afferent sensory nerve fiber. When the muscle contracts it pulls on the capsule, which causes the collagen to squeeze the nerve endings, leading to the generation of action potentials. This response is slowly adapting. In general, one can think of the GTOs as the motor control systems force feedback receptor, or force transducer. It was once thought that these receptors only conveyed information when a muscle was stretched to within its limits, and thus functioned as a warning system. However, this false interpretation came about due to the fact that when passively stretching an entire muscle, such as the biceps, the amount of force across any single muscle unit and its GTOs is rather small. This is in contrast to when that muscle unit is actively contracting. Thus, GTOs relay information preferentially about forces that are actively generated.

Just as GTOs are the force transducers of the motor control system we can think of **muscle spindles** as the motor control system's encoders relaying information on position, velocity and acceleration. Briefly, muscle spindles are comprised of intrafusal (with spindles) muscle fibers, which are connected to extrafusal (without spindles) muscle fibers that are responsible for generating force. The central region of the intrafusal fiber is encapsulated within a fluid filled spindle. Sensory endings make attachments to this non contractile central region of the intrafusal fibers within the spindle, and are stimulated when the fiber is stretched that is when the muscle is lengthened. In addition, outside the spindle region the intrafusal fibers have contractile regions that can be stimulated via the fusimotor system, also called the gamma motor system. This gamma stimulation adds tension to the spindle and can come in two forms, static and dynamic activation that can be used to increase the bias or gain of the spindle.

There are two major types of sensory endings innervating the spindles, Ia (primary) and II (secondary). The Ia afferents are large diameter fast conducting

and convey velocity and acceleration information to a larger extent than position, which is preferentially conveyed by type II afferents that are smaller and conduct action potentials at a slower speed. There are three types of intrafusal fibers within the spindle, nuclear bag 1, which are activated by gamma dynamic input, nuclear bag 2 (static) and nuclear chain fibers that receive static gamma activation. The word nuclear refers to the multiple nucli that these fibers have, and that either line up parallel to the long axis of the fiber (chain) or group together (bag).

When we use our muscles, such as our biceps during elbow flexion, the extrafusal fibers of the bicep contract and bring with them the intrafusal fibers. As stated above, the sensory endings respond when the intrafusal fibers are stretched, not when shortened as in our example here. However, in this case the antagonistic muscles (tricepts) will be stretched and thus its muscle spindles will inform the CNS of this. In addition, the gamma system can be used in a manner called alpha-gamma coactivation. During alpha-gamma coactivation the output of the muscle spindles in the biceps from our example can act as error detectors. As the extrafusal muscles are activated via the alpha motor system and they contract. During this contraction the gamma system activates the contractile region on the intrafusal fibers, keeping them taunt at an expected length, which is the length predicted via the alpha activation. If you happen to be lifting an object and you underestimated its weight then your biceps would have contracted less than expected and due to the aforementioned gamma activity your bicep's muscle spindles will fire indicating this error.

A third type of peripheral receptor is simply called a joint receptor. **Joint receptors** are mechanoreceptors located in joint capsules, and it was once thought they acted as a warning system, signaling the edges of a joints workspace (Burgess and Clark 1969; Clark and Burgess 1975). Others have argued that these receptors signal movement, but not position over much of the joints workspace (Proske et al. 1988). Due to an anatomical oddity it is possible to disengage the muscles from the last segment of your middle finger (distal interphalangeal joint) by simply pointing this finger down while pointing your index, ring and pinky fingers up. Using this hand posture it has been shown that we can use the information from joint receptors alone for proprioceptive feedback; however, our full proprioceptive abilities require the use of our muscle spindles as well (Ferrell et al. 1987).

11.2.2 Central Representations of Proprioception

The representation of proprioceptive information coursing through the spinal cord, dorsal column nuclei, thalamus, cerebellum, and cerebral cortex have classically been studied using electrophysiological techniques employing large electrodes that record EEG ($\sim$10,000 neurons) or local field potential (LFP $\sim$100 neurons) sized neural ensembles, lesioning experiments followed by

behavioral testing, and histological analysis. In addition, studies have been carried out in which tracers have been injection into specific regions within the CNS in order to elucidate the passage of fiber tracts or neural projections. After the introduction of the microelectrode in the 1950s it became possible to record from individual neurons. Advancements in single neuron recordings from whole animal preparations including the awake behaving animal have ushered in a new phase of neuroscience. Below I will briefly cover the literature on the flow of proprioceptive information from the spinal cord to its terminal cortical and sub-cortical projections.

11.2.2.1 Spinal and Cerebellar

Bosco, Poppele and their colleagues have carried out a series of experiments on the cat dorsal spinocerebellar tract (DSCT), which carries the majority of proprioceptive information from the peripheral mechanoreceptors of the hindlimbs. The ventral spinocerebellar tract also conveys proprioceptive information, but is most likely not as purely sensory showing signs of descending motor information (Lundberg 1971; Lundberg and Weight 1971; Arshavsky et al. 1972b; Arshavsky et al. 1972a). Proprioceptive information paralleling the above tracts from the forelimbs is carried via the cuneocerebellar and rostral spinocerebellar tracts respectively (Grant 1962; Oscarsson and Uddenberg 1964). Bosco and Poppele have suggested that the information from the peripheral receptors are already combined within the spinal cord, and that the DSCT is carrying a higher level code representing global information about the limbs rather than simply the length of the muscles, or the angle of the joints. Specifically, they have suggested that this proprioceptive code is in a polar coordinate system with the endpoint (foot) represented as a length from the hip to the paw, and the angle made between the limb axis and the hip Fig. 11.3.

The fact that there are only two degrees of freedom in their coordinate system may have been due to a high degree of coupling among the three joints of the cat hindlimb, and the fact that its hindlimb's motion is mostly restricted to a plane. However, in a set of experiments the natural coupling between the joints was canceled by fixing one joint angle while varying the endpoint position with a similar conclusion about the endpoint representation (Bosco and Poppele 2000; Bosco and Poppele 2003). Recent work from this group has moved past static paw positions and even into active stepping and has suggested that the limb length variable is not actually an independent variable, and encodes limb loading, which they argue is proportional to limb length under their previously explored passive conditions (Bosco et al. 2005; Bosco et al. 2006).

The aforementioned four spinal tracts give rise to mossy fibers that innervate the cerebellum, while the olivocerebellar pathway provides climbing fiber inputs to the cerebellum. These inputs reach the spinocerebellar cortex, consisting of the vermis and intermediate cerebellar cortex, as well as to the interpositus nucleus. The cerebellar cortical representation of this information and

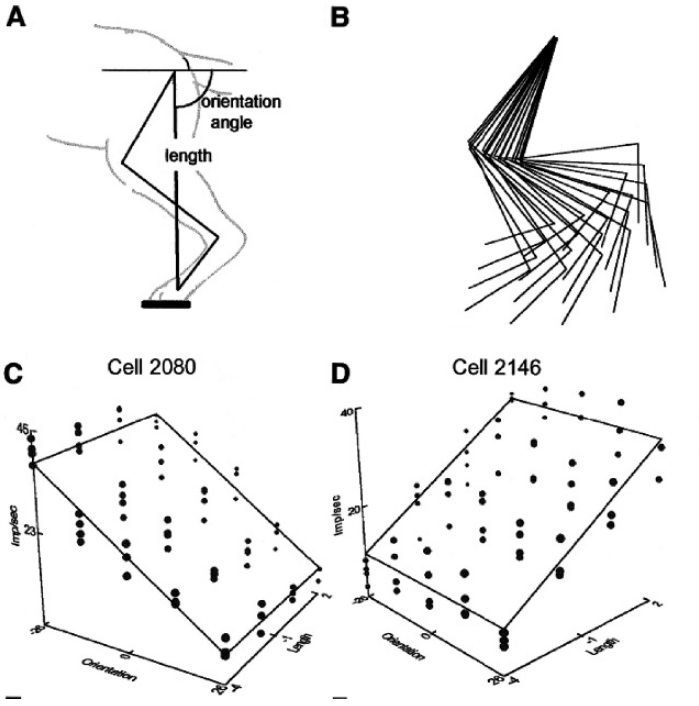

Fig. 11.3 (**A**) The polar coordinate scheme with the hip as the origin. (**B**) The range of 20 foot positions used to test the neural representation from the DSCT. (**C**) and (**D**) represent the activity from two DSCT neurons. The *x* axis is the length, the *y* axis the orientation and the *z* axis the neurons firing rate. (taken from (Bosco and Poppele 2001) with permission)

somatosensory information in general, are in a fractured form, and there is no clean somatotopy. As it has been suggested that the information in the spinocerebellar tracts is already coding high level information, past a single joint or muscle, it should be no surprise that such global limb information is also found in the cerebellum, representing both position and velocity (Casabona et al. 2004). However, the cerebellar representation of limb position dose not seem to be as strong as that seen in the DSCT (Casabona et al. 2003). Interesting is the fact that this positional representation is anisotropic (Valle et al. 2007), which was been described in primate motor (Kettner et al. 1988) and somatosensory cortex (Tillery et al. 1996) as well.

11.2.2.2 Thalamic and Cortical Representation of Proprioception

The adjoining figure (Fig. 11.4) by Jon Kaas et al. illustrates nicely the connections form the spinal cord up to the cortex involved in somatosenstion (Kaas et al. 2002). As seen in the figure, information from peripheral receptors ascends the spinal cord in two major pathways, the dorsal column system and the

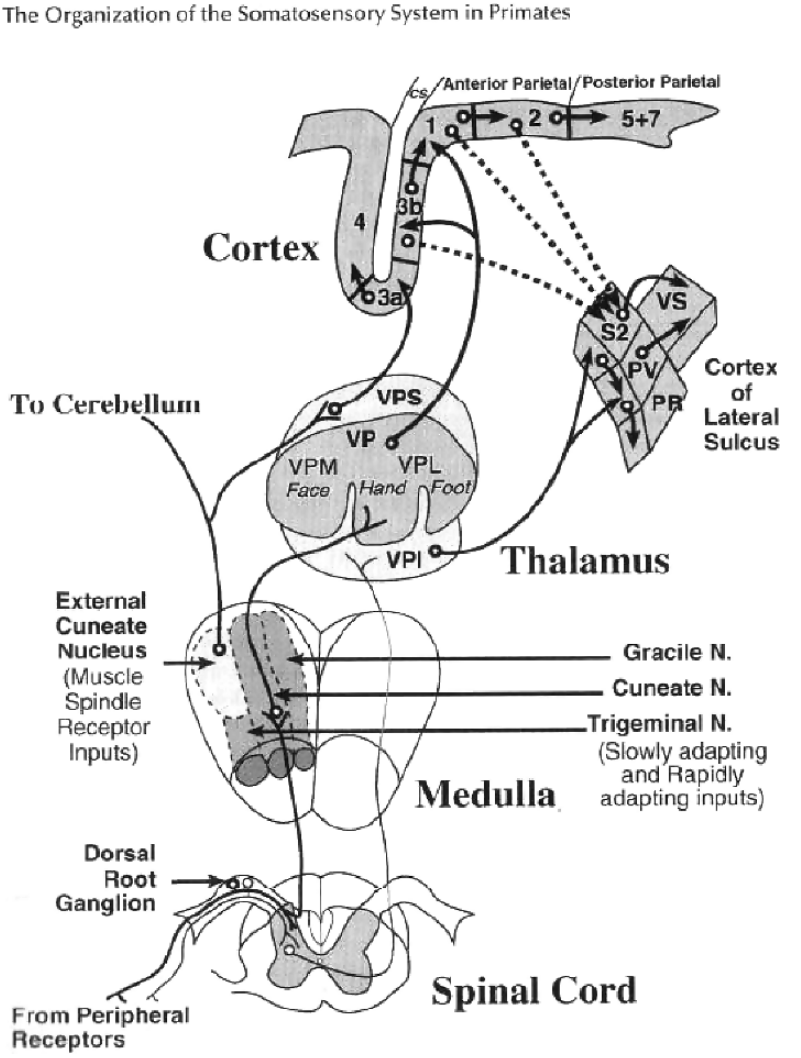

Fig. 11.4 Diagram of the flow of information form the peripheral mechanoreceptors to the cortex. (taken from (Kaas et al. 2002) with permission)

spinothalamic systems. In the monkey the information from these two systems is segregated between the main somatosensory thalamic relay nucli, such that the spinothalamic information is carried to the ventral posterior inferior (VPI) nucleus as well as the VMpo (Craig and Zhang 2006), while information from the dorsal column nucli (DCN) project to the ventral posterior VP (comprised of the VPL and VPM). A majority of information from muscle spindles carried to the DCN terminate in separate nuclei, such as the external cuneate nucleus

for the forelimb, than that of the cutaneous receptors, which send their major projections to the cuneate and gracile nucleus.

The VP has a well defined somatotopy in mammals (Kaas 2007) with the hand and foot occupying a disproportionately large volume, as compared to the rest of the body (Angel and Clarke 1975; Wilson et al. 1999). There are cell poor septa separating the face, hand and foot representations (Welker 1973; Kaas et al. 2002). The VP receives as its primary type of sensory input, from the DCN, both rapidly adapting (RA) and slowly adapting (SA) information, coming from these two main groups of receptors in the skin. The information from these two groups of receptors reportedly synapse on separate populations of VP neurons (Dykes et al. 1981), thus with future advances in micro and even nano electrode technologies, one may be able to differentially stimulate these two populations for use in a somatosensory neuroprosthesis (Rozenboym et al. 2005; Rozenboym et al. 2006). The use of nano electrodes may not be necessary in the human, as a focal stroke in the VPL has been shown to specifically impair static, but not dynamic touch (Timmermann et al. 2000). This data suggests that the SA and RA information is conveyed by separate portions of the VPL. Such modality specific losses have also been shown for proprioception in humans (Sacco et al. 1987).

The VP projects strongly to LAYER IV of 3b, which is considered to be the homologue of S1 in non-primate mammals (Kaas 1983). Receptive fields in area 3b are smaller than in the other somatosensory areas (areas 1, 2, S2, PV), as would be expected of the primary somatosensory cortex (S1). The VP also projects to area 1, predominantly above layer IV. Area 1 receives a major layer IV projection from 3b. Thus, it is possible that VP plays a modulatory role in area 1, while being the source of excitation in 3b. This architecture implies a serial order of information processing primarily from VP to 3b and onto area 1. This apparent serial order of information flow continues onto area 2, which receives input from area 1, but not directly from VP. Area 2 receives its main input from area 1 as well as from the **VPS**, which is the main relay nucleus for proprioceptive information in the monkey. Area 2 may be involved in form recognition by combining cutaneous and proprioceptive information for hapsis.

The **VPI** along with the **VMpo** appear to be the major thalamic recipients of lamina I projections in the macaque (Craig et al. 1994; Craig 2006), whereas the caudal VPL is in the rat (Gauriau and Bernard 2004). It has been proposed that the VMpo is a specific relay nucleus for pain and temperature information. The VPI is the primary thalamic input to S2 and PV, and projects to the superficial cortical layers (Krubitzer and Kaas 1992). This VPI input seems to have a modulatory role rather than directly activating S2 and PV. In fact, if one lesions areas 3a and 3b there appears to be no cutaneous excitation of area 1, S2 or PV (Garraghty et al. 1990a; Garraghty et al. 1990b). We will see later in this chapter that this type of obvious serial order processing doesn't seem to occur in the sensory motor areas during movement planning and execution.

Until recently, the rat homolog of the VPS had not been identified, which is surprising given the wide spread use of the rat as an animal model. We have now

mapped out a region in the rostral VPL of the rat that responds preferentially to joint manipulation and muscle palpation (Francis et al. 2008). This region contains large cutaneous receptive fields on the paws as well (see Fig. 11.5A). Just caudal to this region of the rat thalamus is an area of the VPL that has small

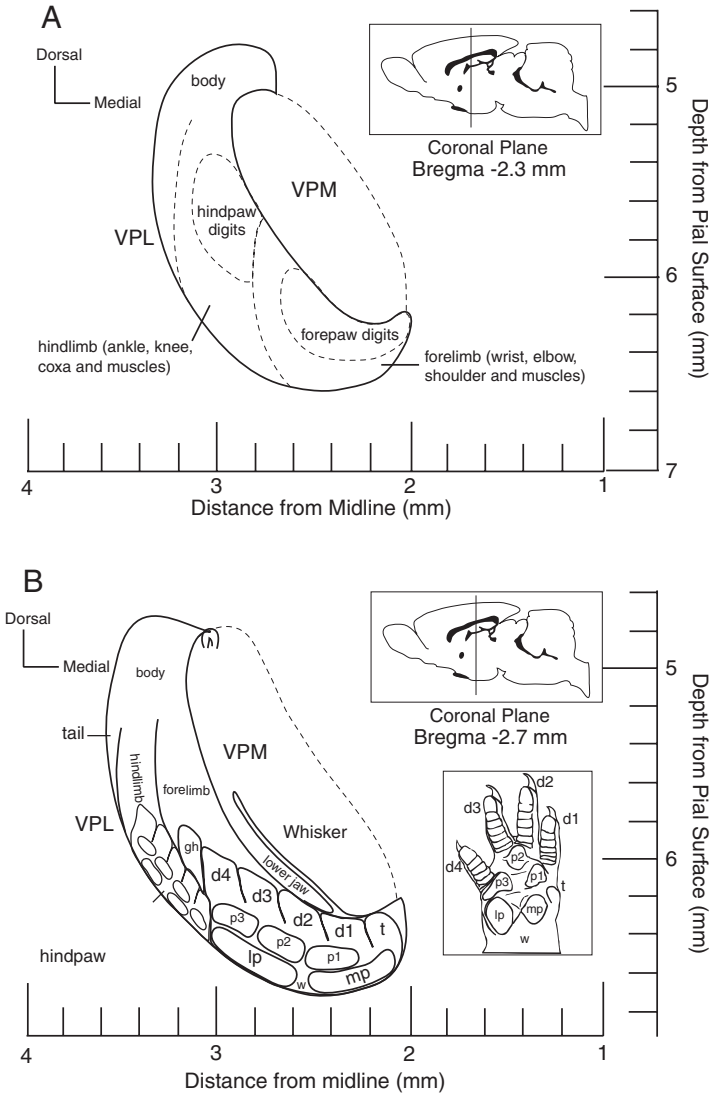

Fig. 11.5 A cartoon of somatotopy of the rat VPL in the coronal plane. (**A**) Bregma –2.3 mm corresponding to the rVPL, and (**B**) Bregma –2.7 mm corresponding to the mVPL. Insets in the *upper right* of (**A**) and (**B**) depict the position of the plane related to the whole rat brain. Inset in the *lower right* of (**B**) depicts the rat right forepaw: lp, lateral pad; mp, medial pad; w, wrist. The depth readings are in reference to the highest point of the pial surface, which is about 0.4 mm below Bregma. (Taken from (Francis et al. 2008) with permission)

cutaneous receptive fields and an exaggerated representation of the fore and hind paws (see Fig. 11.5B). This region corresponds to the primate VPL, and we have termed it the middle VPL (mVPL) in the rat (Francis et al. 2008). Caudal to the mVPL is the cVPL (caudal VPL), which has large cutaneous receptive fields, and may be the rat homolog of the primate VPI/VMpo.

11.3 Neural Representation of Kinematics vs. Dynamics

Does the motor control system use extrinsic (hand position) or intrinsic (joint/muscle based) coordinates, and is it controlling kinematics (position) or dynamics (force) related variables. These questions have been fiercely debated for some time now (Georgopoulos et al. 1982; Georgopoulos et al. 1983; Scott and Kalaska 1995; Crammond and Kalaska 1996; Taira et al. 1996; Ashe 1997; Scott and Kalaska 1997; Scott et al. 1997; Sergio and Kalaska 1997; Ajemian et al. 2000; Todorov 2000; Ajemian et al. 2001; Reina et al. 2001; Sergio and Kalaska 2003; Sergio et al. 2005) (for review see Shadmehr and Wise). In general, it seems that certain brain regions tend toward kinimatics and others toward dynamics, and even within a brain region a given neurons representation may appear to shift. In this section I will present some key results and experiments related to this debate from the primate and rat literature.

Let's start with the spinal cord and muscles and discus some of their properties and control abilities before we move up the nervous system to the brain. Muscles have both active contractile properties as well as passive elastic properties, like a spring, it requires more force to lengthen, or shorten it as you get further away from its preferred length. As a simple example consider the arm configuration of the subject in Fig. 11.1, and assume that we have the subjects arm supported via a sling so they don't have to counter gravity. Their relaxed arm will wish to come to rest at some position that is governed by the passive spring like properties of the muscles, assuming there are no external forces acting on the arm. You could call this the arms preferred (or zero effort) equilibrium position. Thus, if they wish to move their hand to another location and keep it there they need to produce enough force via their muscles to overcome the inertia of their arm as well as overcoming the passive spring like properties of the muscles that resist movement away from their preferred length. This latter component leads to the need for a tonic neural signal activating the muscles to keep them at this new equilibrium position.

In **1966**, Feldman conducted a set of experiments in which he had subjects hold their arm with a given elbow angle and tested what forces the subjects generated at their hand when he tried to move their arm slightly (Feldman 1966). What he found was that the subjects where unknowingly producing restoring forces that resisted this imperceptible movement. This was taken as evidence of an equilibrium point that was set presumably via the brain and instantiated via the spinal cord, the muscles and mechanical receptors. It was

theorized that movements could be produced by shifting this equilibrium point from where the hand was to some target position. This does not tell us, however, what the motor control system is actually controlling. Is the equilibrium position in muscle/force space, or is it in kinematic space, whether that be joint, or extrinsic.

11.3.1 Wrist Movements

Over the past half century neurophysiological motor control studies in the primate have traveled from the wrist and finger movements, up to the elbow and finally to the shoulder and reach and grasp movements. Below I have grouped pertinent papers addressing the control of force and position into the wrist, elbow and whole arm categories.

In **1968** Edward Evarts presented a series of historic papers utilizing a motor control paradigm where monkeys made wrist movements cued via a light to the right or left while encountering constant assistive, resistive, or null torques via a manipulandum (Evarts 1965; Evarts 1966; Evarts 1968). Evarts used electrical stimulation in the medulary pyramids in order to determine if the motor cortical cells he was recording from were pyramidal tract neurons (PTNs) or not. In this series of papers Evarts describes the relation between conduction velocities in PTNs and their recruitment and firing tendencies, with fast conducting PTNs exhibiting phasic increases in activity during movement, while slowly conducting PTNs were active even during posture and could have both increases and decreases in activity during movement. These results point to the possible separation between the control of posture and movement (Kurtzer et al. 2005), which is still an active area of research. In this earliest work that specifically addresses the dynamics (force) vs. kinematics (position) representation in the motor cortex; Evarts suggests that it is the magnitude of the force that is best accounted for in M1. In a follow up paper Evarts looked at PTN activity during a postural task, which is identical to the one he previously used, but now the monkey had to resist movement of its wrist while experiencing the different load conditions. Again, the results pointed to the control of force via the motor cortex (Evarts 1969), where many PTNs changed their activity in relation to the change in force per unit time dF/dt. Evarts also noted that the motor cortex receives input from proprecceptors via Ia afferents that convey information from the nuclear bag fibers of the muscle spindles. Thus, it is conceivable that some of the information represented in the motor cortex is in fact related to muscle lengths and not simply to force. This representation could include both feedforward sensory expectations via the gamma motor system, as well as feedback via the Ia afferent system. This is not to imply that the motor cortex has the leading role in these proprioceptive neural capacities. Another point that Evarts recognized was the fact that the monkey had to hold the handle and this would lead to different combinations of force needed by the

fingers, or thumb depending on what direction the handle was to be moved. Such confounding aspects are common in much of the motor control literature.

In **1980** Paul Cheney and Eberhard Fetz published an influential paper describing the activity patterns of putative corticomotoneurons (CM) in the motor cortex. These neurons demonstrated postspike facilitation (PSF) of the rectified electromyography (EMG) activity. Thus, unlike the PTNs (see above),which could have had targets other than the muscles, or even the spine, these CM cells are chosen based on a relation with the EMG data, this technique is called spike triggered averaging. It should not be surprising that these CM cells, that were selected due to their spike triggered average relation to the EMG, were found to always be active during the production of a torque in the cells preferred direction (direction the cell is most responsive). It was noted that there were four main firing patterns in the CM cells, which are labeled and their numbers shown in Fig. 11.6 taken from (Cheney and Fetz 1980). According to the authors the CM activity was similar between isometric and auxotonic conditions, indicating that the CMs are most related to the torque trajectory, rather than the displacement, or its derivatives. They also noted that the two phasic categories (phasic-tonic and phasic-ramp) of CMs began to fire significantly earlier (–71 and –63 ms, respectively) than the ramp and tonic CMs did (+5 and +101 ms, respectively). Again this separation may represent the neural control for active changes in force output, which are generally associated with movement, and postural forces, or an equilibrium point. In this case the equilibrium point need not be in positional space, but rather in torque output.

These authors found that the relation between the change in torque and CM firing rate was steeper for wrist extension than for flexion, and that in general

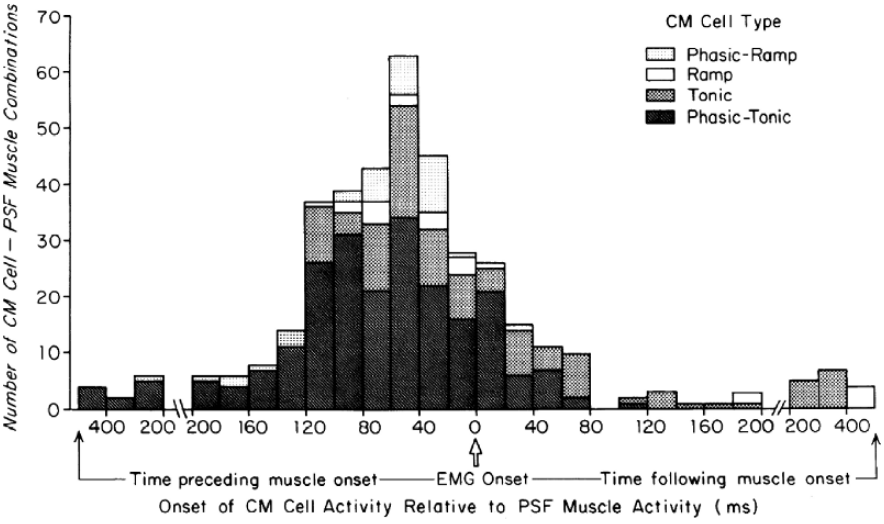

Fig. 11.6 Distribution of the onset of corticomotoneuronal (CM) activity with respect to post spike facilitated muscle activity. (Taken from (Cheney and Fetz 1980) with permission)

when the torque was increased the CMs would increase their rate rather than new CMs being recruited at higher torque levels. Put another way, very few CM cells had a torque threshold that was larger than zero for at least some tonic activity. Others have suggested that there is an extensive representation in M1 around the zero torque, and for the lower torque range, and that the slope of the relation between M1 neural firing and torque change is steepest at the low torque range, at least for the wrist (Werner et al. 1991). Werner and colleagues even suggest that there may be different roles for M1 and the pre-motor cortex in controlling fine vs. gross forces, as they found the PM cells to represent torque at a larger range and these neurons had a steeper force/firing rate relation at higher torques (for force review see (Ashe 1997)).

An early debate in motor control that the above work helped settle had to do with whether the motor cortex controls individual muscles or groups of muscles. Anatomical evidence has indicated that neurons in the motor cortex innervate multiple motor pools (Shinoda et al. 1981), indicating it controls groups of muscles and not just one, further support of this idea came from the above electrophysiological experiments (Cheney and Fetz 1980; Maier et al. 1993), were it was found that the spikes from one motor cortical cell influenced several muscles, and these muscles often worked together.

In **1983** Humphrey and Reed showed that monkeys would use stiffness, or compliance control, for high frequency alternating torques on the wrist. Whereas they would use reciprocal activation patters at lower frequencies. There where two distinct cell populations in M1 for this joint stiffness and forward model type control. Below are results from another set of wrist experiments where distinct cell populations were found to represent specific movement related variables, however, this is most likely due to the specifics of each of these different experimental paradigms as well as the methods used for choosing cells etc. . .

In **1994** Alexa Riehle et al. addressed the issue of controlling force and the extent of wrist movements (Riehle et al. 1994). In this work they utilized a paradigm in which partial or no information about the upcoming movement could be given during a delay period in order to determine the influence this information had on motor planning and execution. Specifically, they would cue information about the upcoming movements force or extent. It had been previously demonstrated that these two variables could be independently controlled, along with movement direction and extent (Rosenbaum 1980), direction and force (MacKay and Bonnet 1990), as well as movement direction and duration (Vidal et al. 1991). Thus, one should be able to find a neural correlate of these, and indeed there are such correlates (Riehle et al. 1994; Riehle and Requin 1995), and the neural representation of these two variables differs from brain region to region, as well as during the evolution of the motor plan and execution. For extent and force these authors found two populations of neurons, one relating only to extent and the other only to force with most of the premovement changes occurring in the pre-motor cortex. These authors suggest that the motor control system programs extent and force in a serial manner, while direction and extent

are planed in either a fixed order, or in parallel, as determined via reaction times using the above task. It may not be suppressing that two distinct neural populations could be found representing either movement direction or movement force. However, unlike some of the previous work it seemed that more neurons changed their activity in relation to direction rather than force. It was noted that "the number of purely direction-related neurons increased, whereas the number of purely force-related neurons decreased from S1 to PA, then to M1 and finally to PM." (Riehle and Requin 1995). Here S1 is the primary somatosensory cortex, PA the parietal cortex and PM the premotor cortex.

More recently a group of researchers attempted to further our understanding on such wrist control by dissociating several possible variables of motor control, such as the muscle activity, the direction of the wrist movement and the direction of movement of the visually controlled computer cursor (Kakei et al. 1999). Unlike the Evarts work, these wrist movements were to eight spatial targets in a 2D plane (center out paradigm), not simply to the right or left. In addition, the monkey grasped the manipulandum in one of three forearm positions, pronated, supinated, or between the two, which corresponds to having the hand with the thumb pointing up. The distribution of change in the preferred direction of motor cortical cells, and for the muscles is shown in Fig. 11.7, were the preferred

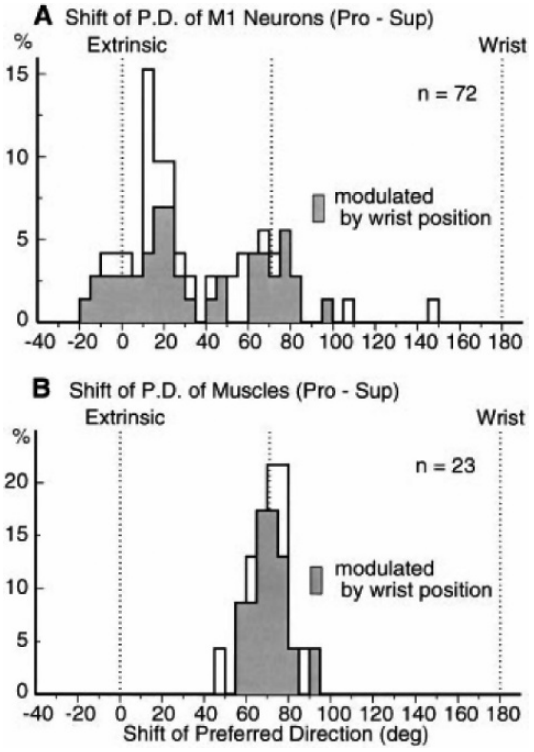

Fig. 11.7 Distribution of the shift in preferred directions of M1 neurons (**A**) and Muscles (**B**) between a pronated and supinated wrist position (see text). (Taken from (Kakei et al. 1999) with permission)

direction is the direction in which the cell or muscle fires most. It can be seen from this figure that the distribution in M1 appears to be bimodal, while that of the muscles is between an extrinsic and wrist centered reference frame, as the difference between the pronated and supinated positions was 180 degree. The authors state that the cortical representation strongly represents both muscle-like and direction of the wrist in space regardless of the muscle activation patterns.

This work has been expanded recently using a very similar task while recording from M1 as well as the spinal cord interneurons (Yanai et al. 2008). In this work the monkeys made isometric wrist torques to visual targets utilizing the center out paradigm, but without wrist movement, just isometric torque. The wrist torques generated in the Yanai work were slower (mean, 670 ms) than the movements in the Kakei work (movement time < 200 ms). Also, the muscle activation patterns were less complex during the isometric paradigm as compared to the Kakei work. These differences may have lead to Yanai et al. stating that they didn't see the same bimodal distribution of M1 neural activity as that seen in the Kakei work, although they did see a consistent shift in the cortical PDs with rotation of the wrist. They noticed a bimodal distribution in the spinal interneurons and interpreted their results such that the motor cortex is operating in an intermediate reference frame between extrinsic and muscle like frames, while the spinal interneurons are already representing a muscle based reference frame. They suggest that cortico-spinal interactions are involved in the coordinate transformation between the M1 intermediate frame and the spinal muscle-like frame.

11.3.2 Elbow Movements

A truly amazing finding was published in **1971** by Fetz and Finocchio utilizing elbow movements and isometric muscle contractions in the primate (Fetz and Finocchio 1971). These researchers determined that monkeys could learn to control the firing patterns of individual motor cortical neurons if they were provided with auditory or visual feedback on the neurons rate of activity. The animals were rewarded for increasing the activity of the neuron, which would normally be associated with the activation of the biceps. However, with training the monkeys could dissociate the muscle contraction from the increased firing of the neuron. The opposite dissociation that is neural suppression while contracting the biceps was incomplete, but still noteworthy. These results are important for two reasons. The first, as pointed out by the authors, is that temporal correlations between neural firing and movement related variables, such as EMG, do not prove that there is a causal relation between the two, as this relation could be controlled by the monkeys. The second reason has to do with the brain machine interface. This work indicates that the individual has the ability to change their neural output from the motor control system, and thus the user of a brain machine interface may be able to control the system even if the translation of neural activity to say robotic motion is less than perfect.

Let's take a look back at the equilibrium point hypothesis using elbow movements in the monkey. Polit and Bizzi trained monkeys to make elbow movements in the horizontal plane to visual targets without vision of their arms (Polit and Bizzi **1979**). The monkeys sat in a primate chair with their right forearm placed in a splint, which was then attached to a one degree of freedom robotic manipulandum that could move in the horizontal plane. This manipulandum was used to track the monkeys elbow angle to determine the precision of his pointing. After the monkeys mastered this pointing task they were deafferented, that is, the dorsal roots of the spinal cord that receive proprioceptive and cutaneous input from the arm and hand were surgically cut (Polit and Bizzi 1979). Even thought these monkeys had no somatosensory feedback from their arms to their spinal cord they could still make these targeted movements, but only while making movements that they had been extensively trained on. If the initial arm configuration was different from that used during training the monkey would make systematic reaching errors. Errors were also induced if a constant torque was produced by the manipulandum. However, short pulse torque perturbations just before movement onset did not fully impair the deafferented animal's ability to make successful movements. Intact animals had no difficulties compensating for each of the above perturbations. One can interpret these results to mean that the monkeys could use feedforward control to make well practiced reaching movements, and the passive properties of the musculoskeletal system are resistant to some perturbation before movement. However, if a constant perturbation interferes with the feedforward plan sensory feedback information is necessary. These results were taken as evidence of the equilibrium point hypothesis, and later work by Giszter, Mussa-Ivaldi and Bizzi demonstrated equilibrium points that could be produced by stimulating electrically in the frog's spinal cord, thus there may be a spinal mechanism for maintaining such points (Giszter et al. 1993). More recently such equilibrium points or postures have been found by using electrical stimulation of the motor and pre-motor cortex (Graziano et al. 2002).

In **1990** Alexander and Crutcher published a series of three papers focused on M1, SMA and the putamen that addressed the neural representation of the intended direction of movement, muscle patterns and the visual target of elbow movements in the primate (Alexander and Crutcher 1990b; Alexander and Crutcher 1990a; Crutcher and Alexander 1990). In this work the monkey made visually guided elbow flexions and extensions with and without constant torque loads. In addition, some experiments dissociated the visual feedback from the actual arm movement such that they were in opposite directions. As this was a delayed movement task they could also quantify the number of neurons from each area that had preparatory activity as well as movement dependant activity.

Alexander and Crutcher found that each of the motor areas tested had activity during the preparatory period, that is during the motor planning, as well as during the movement period. The directionally tuned preparatory activity was either related to the visual information, regardless of the actual arm

movement made, which they called target-dependant cells, or they represented the actual arm movement, in which case they were termed limb-dependent. There were approximately equal numbers of preparatory target-dependant cells in the three brain regions, while the SMA had more preparatory limb-dependent cells (40%) as compared to M1 (15%) and the putamen (9%). Along with these two types of preparatory neural activity there were also the same two types of movement related neural activity, which are target-dependant and limb-dependant activity during the elbow movement time. The majority of these movement related neurons were limb-dependant with 71% in M1, 65% in SMA and 14% in the putamen. As the authors point out, these results indicate that there is a good deal of parallel processing occurring within the motor control system, and the activation patterns do not support a serial passing of information from one region to the next. In general, there were differences in the mean and median peri movement and peri cue activity between the brain regions, such that the neural activity occurred earlier in the SMA then M1 and last in the putamen. However, the distributions of neural activity around the onset of movement for each region spanned both positive and negative time lags, that is they could be preparatory (motor planning, or sensory expectation), or sensory/feedback like. Each of these three areas also had neurons with short latency proprioceptive like responses and muscle like responses that represented the different torque loads, with these activities being more prevalent in M1.

11.3.3 Whole arm Movements

Over the past two decades a group of researchers led by, or former students and post-docs of, Apostolus Georgopoulos have contributed tremendously to our current options on the motor control system. In **1982** Georgopoulos et al. reported that the neural activity of about 39% of M1 cells (323 cells out of 606) changed their neural firing in a direction dependant manner, and that this relationship between the direction of movement and the cells rate of activity could be accounted for via a cosine function. The behavioral paradigm utilized was a center out reaching task (2D) performed while the monkeys held the handle of a planar manipulandum. An important idea that comes out of this work is that the motor cortex has a distribution of neurons that code for the direction of movement in a smooth manner, with each cell having a preferred movement direction that it fires most in with this firing rate dropping off smoothly via a cosine function of the difference between the current movement direction θ_d and the cells preferred direction θ_{pd} , such that $F(d) = b_o + c_1 \cos(\theta_d - \theta_{pd})$, where F is the neural firing rate, d the current direction of motion, b_o the mean firing rate, c_1 a gain factor and pd stands for the cells preferred direction (Georgopoulos et al. 1982). Similar neural tuning

was subsequently shown when the monkey held its hand in a static posture at each of the outer targets in the center out paradigm (Georgopoulos et al. 1984).

In 1986 this cosine tuning idea was taken to the population level, that is, how can neurons with broad tuning curves specify accurately a movement to a given direction in 3D space? A solution to this problem was termed the population vector (Georgopoulos et al. 1986). In essence what the population vector formalism states is that each of the directionally selective M1 neurons contributes information about the current movement direction. The information from all of these cells is summed to form an accurate prediction of the actual movement direction to be made, or that is being made. The amount of information added to the sum by each cell is dependant on that cells preferred direction and the direction of the movement being planed, or made, following the aforementioned cosine directional tuning. John Kalaska et al. combined the center out reaching task (Georgopoulos et al. 1982) with a loaded manipulandum that could pull the monkeys hand/arm in eight different directions. Thus they could now determine the neural correlations to both movement direction as well as load direction. They found that shoulder joint related M1 neurons could have both a preferred movement direction as well as a preferred load direction, which they called the cells load axis (Kalaska et al. 1989). Almost ten years later prudd'home and Kalaska presented similar types of neural activity in S1 to load direction, as noted above for M1, but this S1 activity was less robust in its response to the loads during the target hold time. Area 5 of the parietal cortex demonstrated no appreciable load dependant activity (Prud'homme and Kalaska 1994; Hamel-Paquet et al. 2006).

In an effort to determine if arm orientation could influence the neural representation during reaching movements, Stephen Scott and John Kalaska had monkeys make these center out reaching movements using either a natural arm orientation, or an abducted orientation while holding a manipulandum. They found that a large portion of cells changed their activity from the one posture to the other, either in their tonic activity, the directional tuning as reviewed above, or both. These changes were seen during the movement time as well as during the postural maintenance of the target hold period (Scott and Kalaska 1995; Scott and Kalaska 1997; Scott et al. 1997). These researchers showed that during these two different postures the population vector pointed in different directions, and thus the motor cortex dose not simply represent the spatial trajectory of the hand, but includes at least some information on the arm orientation, and from the previously reviewed results, some representation of force, and proprioception.

The above work involved primates grasping and moving a manipulandum and thus any loads generated via the manipulandum had to be represented at the handle. To overcome this situation and test the force related neural activity at the shoulder and elbow during reaching movements Stephen Scott introduced the use of an exoskeletal robotic manipulandum (KINARM). It was noted that equal numbers of M1 neurons were sensitive to torques applied via the KINARM to the shoulder, elbow, or both, and most cells were related to

flexor torques at one joint and extensor torques at the other. Also, the neural activity to combined shoulder and elbow torques was predictable based on the neurons response to the two individual torques via a vector sum (Cabel et al. 2001). A second paper from this group in 2001 showed systematic discrepancies between the M1 population vector's prediction of hand direction and the actual hand direction, and that these discrepancies were due to a non-uniform distribution of preferred directions in the M1 population. Furthermore, the non uniform distribution could be related to the peak joint power at the shoulder and elbow.

As should be clear by now, researchers are steadily finding new correlations between movement related variables and neural activity in the motor cortex, as well as other movement related brain regions in the primate.

As primate research is fairly expensive and it takes a great deal of time and effort to train monkeys, one might wish to conduct some of this motor control research on rodents, and indeed much has been done in the realm of reaching movements (Whishaw and Pellis 1990; Whishaw et al. 1991; Whishaw 1996; Ballermann et al. 2000; Kargo and Nitz 2003; Kargo and Nitz 2004). However, most of this work involved rats reaching for food pellets and did not separate kinimatic and dynamic related variables. In a recent set of papers I have introduced a simple torque manipulandum reaching task for rats (Francis and Chapin 2004; Francis and Chapin 2006). Unlike previous rat lever paradigms where the rat can simply step on a lever with its fore paw, this task requires the rat to actually grasp the manipulandum handle and pull or push it to a specific target position within a given time window, which they learn to do within an hour. Once the rats had practiced making reaching movements for a week or so they were implanted with arrays of recording electrodes in their sensory and motor cortex as well as the proprioceptive thalamus rVPL (Francis et al. 2008). I was then able to use the simultaneously recorded neural activity from these brain regions to predict the time varying position, work and force produced by the rat at the hand. This work has helped close the gap between a simple rat model and the primate motor control experiments that I have reviewed. I plan to take this paradigm into three dimensional movements in the near future. There are some interesting differences between the rat and the primate, the most obvious is that we are heavily dependant on our vision for determining targets of interest and while making reaching movements. However, rats most likely cannot see their hands in a large portion of their personal workspace. It has been suggested that rats mainly use olfaction for determining the position of reaching targets. In my manipulandum task it appears as though the rats are using both olfaction and to a large extent their whiskers to "see" the handle within their whisker field. Unlike the food pellet reaching task, which places the food item outside the animals whisker field, the manipulandum on the other hand is in the animals workspace, so if it wishes it can sniff, lick, bite or grasp the handle. Their first instinct is to bite it, but this can be easily overcome by only allowing the maniplulandum to be moved when they grasp it with their hand (for details see (Francis and Chapin 2004)).

An important concept that I have been probing with these rat experiments, and in companion primate experiments, is the degree of generalization we can determine between reaching movements made in different dynamical environments (Donchin et al. 2003; Francis 2007). This work will be necessary for developing a neural controlled prosthetic arm, so as to allow the user the ability to control their force output when picking up novel objects etc... I have found that as long as I train my neural decoding algorithms on a large enough sampling of force related state space that I can then accurately predict the position, work, and force being produced by the rat, even if they are working against loads not used for building the decoding model. In Fig. 11.8A. I have plotted the variable work (force * displacement) during these rat reaching movements against two different constant loads, one of 3 g and the other 10 g, also plotted are the neural predictions of the work. Fig. 11.8. B. shows the two different forces used as well as the neural prediction of them.

In Fig. 11.9A. I have plotted the cross correlation between the neural prediction of the labeled movement related variable's and the actual variables values for each data set from several animals (Francis and Chapin 2006).

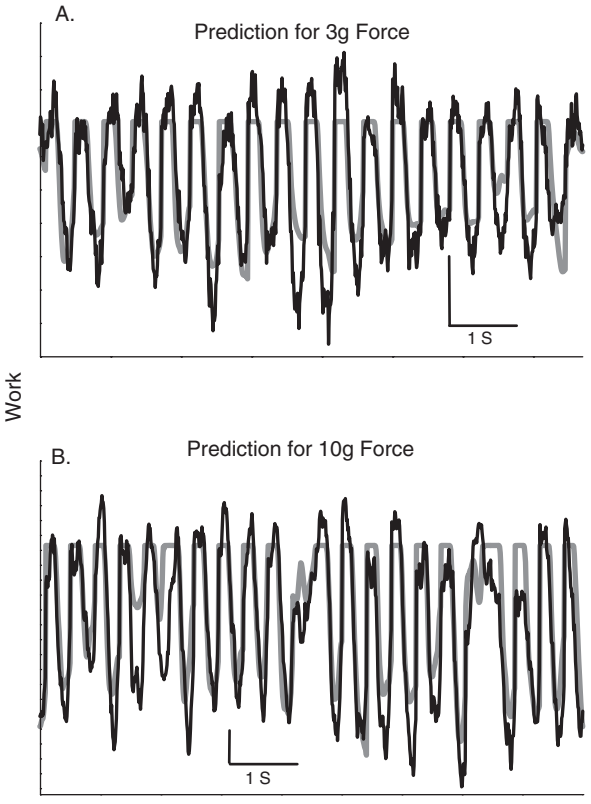

Fig. 11.8 In panels A and B we have plotted the work needed to move the manipulandum in *gray* and the regression model prediction from the neural data in *black*. Note scale differences between A and B. The prediction to the work variable was r = 0.89 and r = 0.87 for prediction of the position variable (data not shown). (From (Francis and Chapin 2006) with permission)

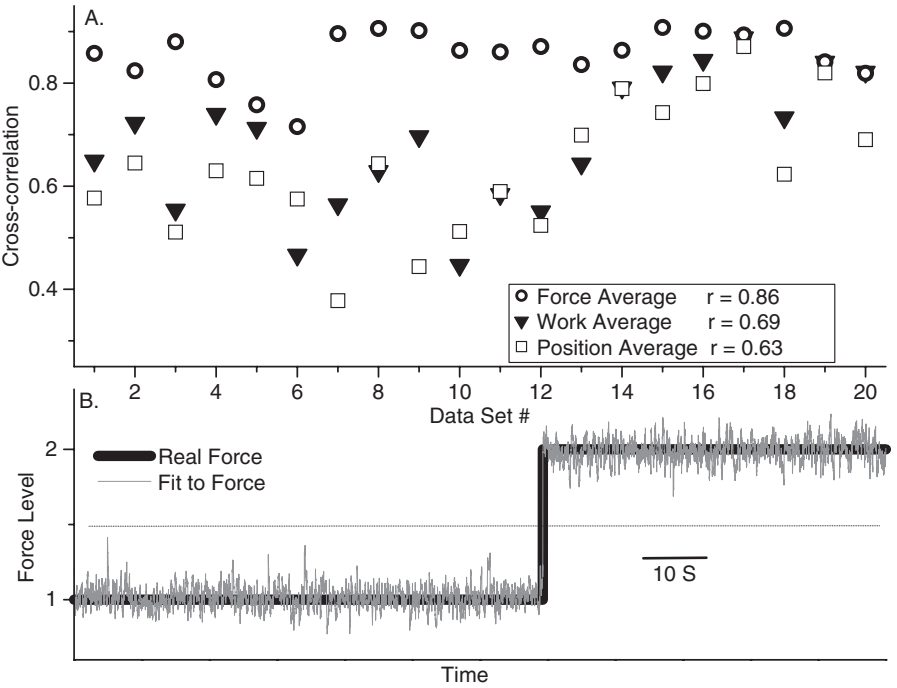

Fig. 11.9 In panel A we have plotted the cross correlation values between each of the three variables of interest's real values and those for the predictions of the models. In panel B we have plotted the real force state produced by the manipulandum as a binomial variable of either 1 or 2 was well as the model's fit to this data. (From (Francis and Chapin 2006) with permission)

Below this plot in Fig. 11.9B is an example of the neural prediction to the force variable during this two force rat reaching paradigm, which was run as a block paradigm. For presentation purposes I have aligned all of the low force time bins to the left and the high force time bins to the right, even thought they were presented in random blocks (Francis and Chapin 2006). To date I have had rats work against 6 different force loads in one experimental session, including 2 constant loads, 2 velocity dependant loads and 2 spring-like loads to allow the dissociation between kinimatic and dynamic variables with similar results to those presented in Fig. 11.8, which are being prepared for publication. I believe that this experimental strategy should allow us to produce a BMI that gives the user control over both positional and force related variables. We are also starting to use the BMI as an experimental tool to ask questions about the neural representation of movement related variables by allowing the animal direct neural control over different types of variables in order to see which ones they are best at transferring to. The BMI is most likely the next great experimental paradigm that will lead to an explosion of new ideas.

11.4 The Brain Machine Interface as a Tool for Motor Control Research

In 1999 a group of researchers led by John Chapin demonstrated that they could record neural ensemble activity, using arrays of microwire electrodes, from the rat sensory-motor cortex, as well as the VL thalamus, and formed a set of mathematical models that would predict the position of a lever being pressed by the rat, which caused a robotic system to deliver a water reward. After the prediction of the models was sound the animal was transitioned to brain control at which time the neural ensemble activity was controlling the robotic motion. After continued practice in brain control mode the animals decreased the number of, or stopped making overt movements, a phenomenon seen by other research teams (Serruya et al. 2002; Carmena et al. 2003). In 2000 a group led by Miguel Nicolelis took the BMI from the rat and implemented it with non-human primates in an open loop paradigm (Wessberg et al. 2000) where they translated neural activity from 3-D reaching movements into 3-D robotic motion. They found very similar results using simple linear models and artificial neural networks, and noted that they needed to continually update their models, otherwise the open loop prediction and robotic control would deteriorate over time.

In 2002 two research teams published results demonstrating that monkeys could use a closed loop BMI, or BMIc, in order to control a computer cursor (Serruya et al. 2002; Taylor et al. 2002) in 2 or 3 dimensional reaching movements. There are some important differences between these two papers. The group of Taylor, Helms Tillery and Schwartz used a modified population vector algorithm (Georgopoulos et al. 1986) with the closed loop result of poor initial control by the animals, so much so they found the results were just as good if they started their incrementally updating algorithm with random weights. This may be due to the fact that the population vector formulism makes certain assumptions about just what the sensory-motor cortices are coding (Georgopoulos et al. 1982; Kalaska et al. 1983; Moran and Schwartz 1999a; Schwartz and Moran 1999). However, after the algorithm had time to update they obtained impressive results. The second group of researchers led by John Donoghue did not make assumptions about the neural code, or at least did not use the assumptions made in the population vector algorithm, but rather used a simple linear regression fitting the neural activity to kinematic endpoint variables such as hand position with good results from the beginning of brain control. However, neither group incorporated the control of force related variables, or of an actual robotic system.

In 2003 the group led by Miguel Nicolelis expanded their earlier work with some important additions. Jose Carmena et al. incorporated a reach to grasp task with a robotic system that the monkey could control in a closed loop manner by moving a cursor on a computer screen that coded for the grip force as the size of the cursor, that is it would increase in size with grip strength

output. This was the first time that force was explicitly introduced into a BMI with successful results using simple linear models (Carmena et al. 2003). However, in this work the animal did not have control over the forces that moved the robotic system, or cursor. In the above cases the monkeys did not actively interact with the robotic system either.

Recently the group led by Andrew Schwartz has demonstrated that a monkey can use an anthropomorphic robotic arm that was positioned at the animals shoulder, so that it acted like the monkeys own arm, to retrieve food items and bring them to the monkey's mouth indicating the possibilities of BMIc (Schwartz et al. 2006). Once again the monkey did not have control over the forces of the robot, but this was the first demonstration of a monkey actually interacting with a robotic system in order to achieve a natural goal with a BMIc.

In 2004 we witnessed two highly publicized instantiations of BMIs in humans. The group led by Miguel Nicolelis used arrays of 32 electrodes that were driven into the thalamus (VOP/VIM or STN). The subjects then interacted with a "video game" in which they had to hold a squeeze ball and produce a given force via squeezing the ball, not that dissimilar to one of the force tasks performed by the monkeys in the Carmena paper. However, in this work they could only record a given site for 5 min, do to the fact that this work was done during DBS implantation surgeries, limiting the amount of data used for fitting and subsequent time for prediction. This work furthered research that has been conducted by PR Kennedy who demonstrated the capacity for a BMI/BCI using a neurotrophic electrode (Kennedy et al. 2000). The group led by John Donoghue benefited from an electrode array consisting of 100 contact points that had been developed by Richard Norman's group (Campbell et al. 1989; Campbell et al. 1990) that has been approved for human implantation. Using this system the Cyberkinetics company implanted a paralyzed individual who could use his neural activity to play video games as well as operate a TV and computer cursor, which has been covered in the popular press. Although this work did not incorporate robotic motion or dynamics it certainly has pushed forward the BMI/BCI capacity in humans and hopefully will help perfect the surgical implantation of such electrode arrays. This work also demonstrates that the motor areas of a paralyzed individual can still be used to control at least kinematic variables such as cursor position.

11.5 Conclusion and Perspective

As we have seen the neural activity in the movement related brain regions appear to represent more than one simple variable, with regions around the central sulcus having both kinimatic and dynamic like representations (PMd, PMv, SMA, M1, 3a, 3b, 1 and 2). The timing of this movement related activity has different distributions between many of the sensory motor control regions, however, several of these regions do have some activity before initiation of

movement, during movement, and even postural aspects that remain after movement, such as a direction dependant hysteresis (Prud'homme and Kalaska 1994). In a recent paper it has been suggest that we look for the representation of spatiotemporal movement related variables, such as preferred pathlets rather than the preferred direction, or any other instantaneous representation, which may prove fruitful (Hatsopoulos et al. 2007).

Over the past few years we have seen an explosion in the number of publications on brain machine interfacing (BMI), and it should now be apparent that such technology can be used to ask fundamental questions about the sensory motor control system. By giving animals and humans real time neural control over different movement related variables while having a robotic system directly move the subjects arm, or simply allowing their neural activity to move a visual cursor, we may be able to further tease apart just what the different brain regions are computing during the different stages involved in motor control. In essence one can think of the BMI as the evolution of the robotic manipulandum.

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Chapter 12
Mechanical Properties of Brain Tissue: Characterisation and Constitutive Modelling

J.A.W. van Dommelen, M. Hrapko and G.W.M. Peters

Abstract The head is often considered as the most critical region of the human body for life-threatening injuries sustained in accidents. In order to develop effective protective measures, a better understanding of the process of injury development in the brain is required. Finite Element (FE) models are being developed, in order to predict the mechanical response of the contents of the head during impact. To obtain accurate predictions of the mechanical response of the brain, an accurate description of the mechanical behaviour of brain tissue is required. However, up to now no universally accepted data set for the constitutive response of brain tissue exists. The large variation in material properties reported may be caused by differences in testing methods and protocols used. An overview of studies on the mechanical properties of brain tissue is presented, focusing on testing methods. Furthermore, the large strain mechanical response of brain tissue as well as modelling approaches for this behaviour are discussed.

Keywords Brain Tissue · constitutive model · mechanical properties · rheology · viscoelasticity

12.1 Introduction

12.1.1 Traumatic Brain Injury

According to data of the World Health Organisation for 2002, road traffic deaths accounted for 23% of all injury deaths worldwide (Peden et al., 2004), see Fig. 12.1(a). In particular, traumatic brain injury caused by a mechanical insult on the head causes high mortality and disability (Brooks et al., 1997; Waxweiler et al., 1995). Despite the development of injury protection measures (belts, airbags, helmets), and increase of governmental regulations, traffic accidents were still responsible for about 40% of all traumatic brain injury

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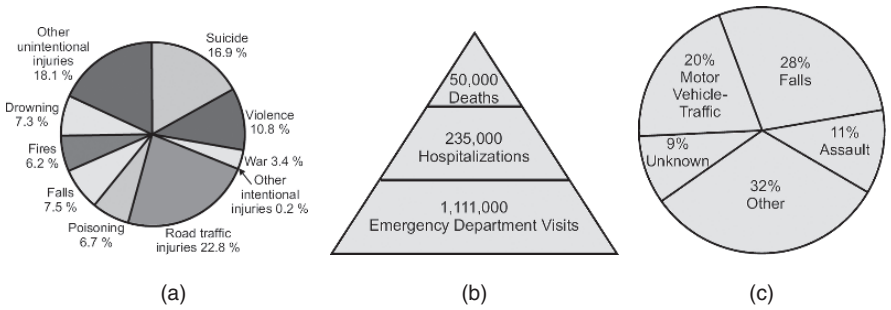

Fig. 12.1 (**a**) Distribution of global injury mortality by cause (Peden et al., 2004), (**b**) average annual number of traumatic brain injury-related emergency department visits, hospitalisations, and deaths in the US in 1995–2001; (**c**) by external cause (Langlois et al., 2004)

(TBI) cases in Sweden in 1997 (Viano et al., 1997). The social costs of these accidents were estimated at 160 billion euro per year in the European Union alone (ETSC, 1999). From 1995 to 2001, an average of 1.4 million cases of TBI occurred in the United States each year (see Fig. 12.1(a) and (b)), of which 20% resulted from motor vehicle accidents (Langlois et al., 2004). Other major causes of TBI are sports (e.g. soccer, boxing, ice hockey, rugby, and American football) and falls. About one third of the hospitalised victims suffer from permanent disability with an inherent high social cost.

12.1.2 Criteria for Head Injury

The occurrence and consequences of TBI can be reduced by the development of improved injury protective measures. The application of macroscopic biomechanical models for injury has already led to a substantial decrease of head injuries. To assess the likelihood of head injury, in the early sixties, the Head Injury Criterion was developed (Versace, 1971; Henn, 1998):

$$HIC = \left\{ (t_2 - t_1) \left[\frac{1}{(t_2 - t_1)} \int_{t_1}^{t_2} a(t) \mathrm{d}t \right]^{2.5} \right\}_{\max}, \tag{12.1}$$

in which $a(t)$ denotes the translational head acceleration in g's as a function of time and t_1 and t_2 represent the initial and final times of an interval that maximises this function. Although this criterion is still used in most current test standards, it suffers from a number of drawbacks, one of which is that it is based on linear head acceleration only. Moreover, it does not allow for a distinction between different injury mechanisms.

Recent safety research focuses on so-called next-generation injury assessment tools for use in future regulations for injury protection devices. During a

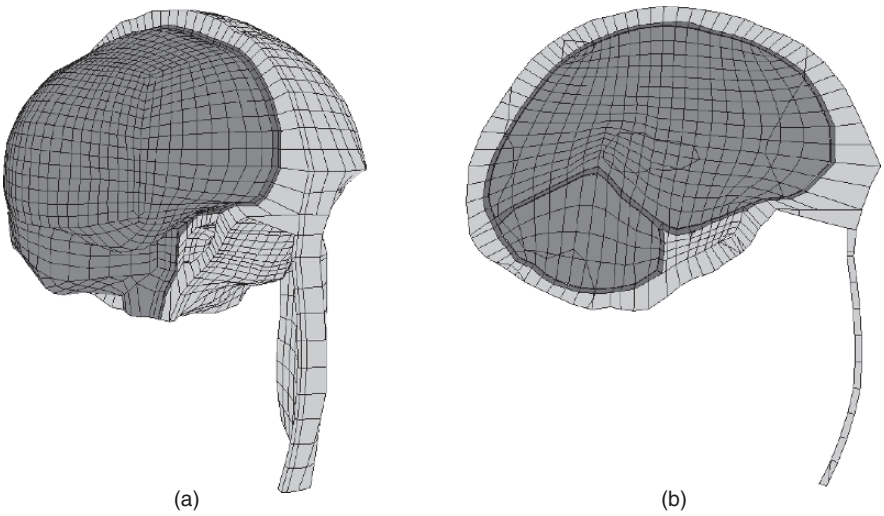

Fig. 12.2 Numerical head model (Claessens et al., 1997; Brands, 2002) with (**a**) exposed brain and (**b**) para-sagittal cross-section

crash the head is exposed to external mechanical loading which causes an internal mechanical response of the brain tissue. Above a certain strain or a strain rate, damage of the brain tissue can occur. Finite Element (FE) models are being developed, in order to predict the mechanical response of the contents of the head during impact. An example of a numerical head model is shown in Fig. 12.2. Current FE head models contain a detailed geometrical description of several anatomical components of the head but often lack accurate validated descriptions of the mechanical behaviour of brain tissue. Without an accurate representation of the constitutive behaviour of the various components, the predictive capabilities of head models may be limited.

In this article, an overview of studies on the mechanical response of brain tissue presented in the literature is given, focussing on different testing conditions. Various aspects are illustrated with the findings and results of the authors. First, some general aspects of testing soft tissues are discussed and some basic concepts of constitutive modelling and characterisation of materials are introduced.

12.2 Constitutive Response of (Biological) Materials

12.2.1 Constitutive Models

Finite element modelling is a powerful tool for the prediction of head injury in impact situations. Simulations with finite element head models require constitutive descriptions for the various materials that constitute the human head.

A constitutive model deals with the relation between the deformation (history) applied to a material and the stress that results from this deformation, which can formally be written as:

$$\boldsymbol{\sigma}(t) = \boldsymbol{\sigma}(\boldsymbol{F}, \tau | \forall \tau \leq t), \tag{12.2}$$

where $\boldsymbol{\sigma}$ represents the Cauchy stress tensor as a function of time t and $\boldsymbol{F}$ is the deformation gradient tensor.

The mechanical behaviour of biological tissue is the result of the properties of the individual microstructural components that constitute the material and the interplay between these components. Some attempts have been made to develop constitutive models for biological materials based on microstructural approaches. However, in impact simulations, mostly phenomenological material models are used. In general, properties of biological tissues are viscoelastic (i.e. their response is rate-dependent and they show stress relaxation at a constant strain level, see Fig. 12.3), non-linear, and anisotropic due to the specific microstructure (e.g. consisting of an arrangement of fibres and surrounding matrix material).

A viscoelastic material model can be represented by a mechanical analogue consisting of a certain arrangement of springs and dashpots. By placing a number of so-called Maxwell elements in parallel, a general viscoelastic framework can be obtained. A differential formulation could be favoured over an integral formulation for the development of a non-linear viscoelastic model because of the possibility to derive it from physics and since it is a numerically more advantageous formulation. A general Maxwell-type differential constitutive equation can be written in the form:

$$\boldsymbol{\sigma} + \lambda \overset{\nabla}{\boldsymbol{\sigma}} + \boldsymbol{f}(\boldsymbol{\sigma}, \boldsymbol{D}) = 2\eta_0 \boldsymbol{D}, \tag{12.3}$$

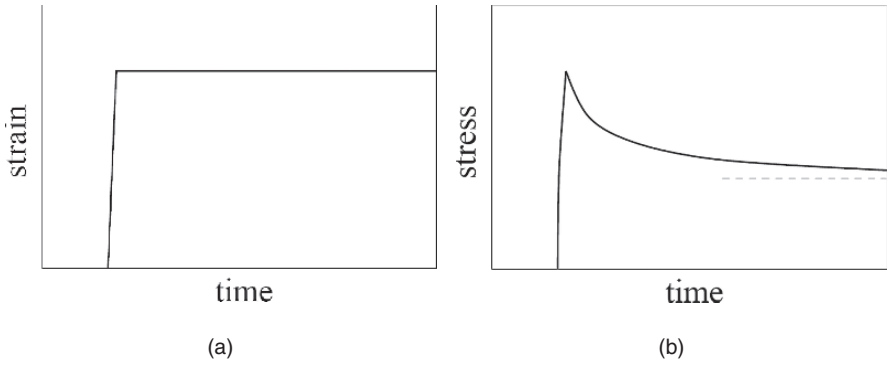

Fig. 12.3 Viscoelastic response to an approximate step in the strain

where now $\boldsymbol{\sigma}$ is the extra stress tensor (that differs from the Cauchy stress by a hydrostatic term), $\boldsymbol{D}$ is the deformation rate tensor and $\overset{\nabla}{\boldsymbol{A}} = \dot{\boldsymbol{A}} - (\vec{\nabla}\vec{v})^{\mathrm{T}} \cdot \boldsymbol{A} - \boldsymbol{A} \cdot (\vec{\nabla}\vec{v})$ represents the upper convected time derivative. In case $\boldsymbol{f}(\boldsymbol{\sigma}, \boldsymbol{D}) = \boldsymbol{0}$, this equation reduces to the upper convected Maxwell model and for small strain amplitudes, a linear viscoelastic model remains.

A linear viscoelastic model can also be written in an integral form, where the concepts of proportionality and superposition are considered. In this theory, the response to an arbitrary loading-history is assumed to be given by a Boltzmann integral over an infinite number of small steps, which can be written as:

$$\boldsymbol{\sigma}(t) = \int_{-\infty}^{t} G(t-\tau) 2\boldsymbol{D} \mathrm{d}\tau \quad \text{with} \quad G(t) = G_{\infty} + \sum_{k=1}^{N} G_k \mathrm{e}^{-\frac{t}{\lambda_k}}, \tag{12.4}$$

with G_{∞} the equilibrium shear modulus of the material. The so-called quasi linear viscoelastic (QLV) theory has been proposed by Fung (1981). This theory has become widely used in injury biomechanics and has been applied for the constitutive or structural modelling of many soft biological tissues (e.g. Woo et al., 1991; Funk et al., 2000; Takhounts et al., 2003). The QLV theory is a generalisation of the linear viscoelastic theory and is also formulated in terms of a convolution integral:

$$\boldsymbol{\sigma}(t) = \int_{-\infty}^{t} M(t-\tau) \frac{\partial \boldsymbol{\sigma}_{\mathrm{e}}}{\partial \tau} \mathrm{d}\tau \quad \text{with} \quad M(t) = M_{\infty} + \sum_{k=1}^{N} M_k \mathrm{e}^{-\frac{t}{\lambda_k}} \quad \text{and} \quad M(0) = 1. \tag{12.5}$$

In this integral representation, the elastic response is separated from the relaxation function. The quasi linear viscoelastic theory assumes the time-dependent behaviour to be given by a Prony series. However a non-linear relation for the instantaneous elastic response to a deformation step can be used. In three dimensions, this instantaneous elastic response is commonly derived from a strain energy function.

12.2.2 Characterisation of Constitutive Properties

A full characterisation of the constitutive behaviour considers the response in various deformation modes (shear, uniaxial tension, compression, biaxial deformation, etc.) and complex loading paths (e.g. reverse loading). Furthermore, the use of constitutive models for biological materials in impact biomechanics simulations requires a characterisation of these materials at high strain rates. The viscoelastic characteristics are typically determined in (small strain)

oscillatory experiments, stress relaxation experiments, and constant strain rate tests at varying strain rates. Prior to the characterisation experiments, specimens are often preconditioned. During preconditioning, the material is subjected to a number of loading–unloading cycles which is aimed at altering the material properties to a state that gives reproducible results and/or is representative for the in-vivo behaviour.

The linear viscoelastic properties of a material are expressed in the parameter sets (E_i,λ_i) or (G_i,λ_i) for each viscoelastic mode i, with E the Young's modulus and G the shear modulus of a material and λ the associated time constant. These parameters can be obtained by direct fitting to stress relaxation experiments. Alternatively, often oscillatory tests are conducted in which a sinusoidal shear (or compressive) strain in the linear regime is applied:

$$\gamma = \gamma_0 \sin(\omega t). \tag{12.6}$$

Viscoelastic materials such as soft biological tissues generally show a sinusoidal shear stress response with a phase shift:

$$\tau = G^* \gamma_0 \sin(\omega t + \delta), \tag{12.7}$$

where G^* is the dynamic shear modulus and δ denotes the phase angle. For fully elastic materials, this phase angle vanishes whereas for purely viscous materials it equals $\frac{1}{2}\pi$. The linear viscoelastic behaviour is then characterised by determining the relations $G^*(\omega)$ and $\delta(\omega)$ for a material. Alternatively, also the storage modulus G' and the loss modulus G'' can be determined, which are defined as:

$$G' = G^* \cos(\delta) \quad \text{and} \quad G'' = G^* \sin(\delta), \tag{12.8}$$

respectively. The functions $G'(\omega)$ and $G''(\omega)$ can both be expressed in terms of the viscoelastic properties (G_i, λ_i).

Characterisation experiments are often conducted in-vitro, on small homogeneous specimens, either in compression/extension or shear, see Fig. 12.4. Shear deformation is often applied in a rotational configuration (in contrast to a translational setup). For a rotational rheometer, a conventional centred geometry will produce a strain distribution ranging from 0 to γ_R at the sample edge. A more homogeneous strain distribution will be obtained if an eccentric sample geometry is used (van Turnhout et al., 2005), see Fig. 12.5. In this configuration, the sample is placed at the edge of the bottom plate, leading to an increase of the measured torque. Moreover, an approximately homogeneous shear field is obtained. In the eccentric rotational shear configuration, the shear stress τ and shear strain γ are calculated from the measured torque M and angle θ by:

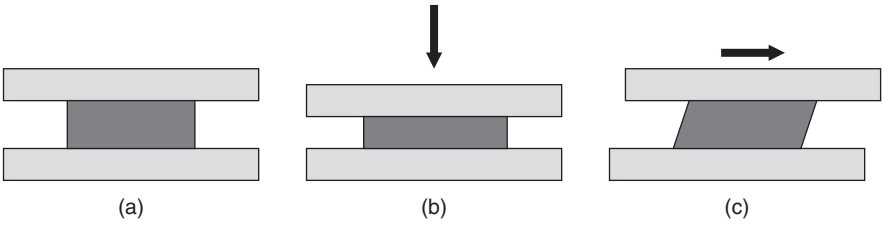

Fig. 12.4 (**a**) Initial configuration, (**b**) compression, (**c**) shear

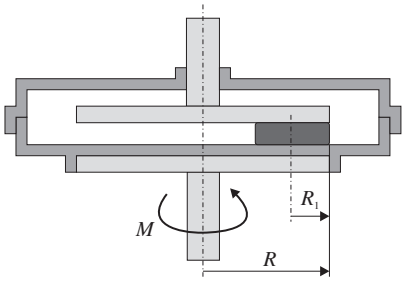

Fig. 12.5 Eccentric configuration for rotational shear experiments

$$\tau = \frac{MR}{2\pi R_1^2\left(\frac{(R-R_1)^2}{2} + \frac{R_1^2}{8}\right)}; \quad \gamma = \theta\frac{R}{h}, \tag{12.9}$$

where R is the radius of the plate, R_1 is the sample radius and h is the sample height. It is assumed that the effect of the free boundaries can be neglected since the sample thickness is much smaller than the sample diameter.

Injury will develop if the mechanical response (e.g. strain, stress, etc.) attains a level at which either the structural integrity of the materials is affected or functionality is reduced. The latter may be the result of physiological processes that occur after the impact, at time scales that are much larger than the time scale of the loading conditions. In case of immediate loss of structural integrity, this will affect the constitutive behaviour of the tissue as well.

12.3 Characterisation of Brain Tissue: The Influence of Test Conditions

Researchers have been studying the material properties of brain tissue using a variety of testing techniques. However, the reported mechanical properties, such as the storage modulus (G') and loss modulus (G''), describing linear viscoelastic behaviour are orders of magnitude different, see Fig. 12.6. Also a large variation in results from stress relaxation and constant strain rate experiments can be observed, see Fig. 12.7. This may be caused by the broad range of

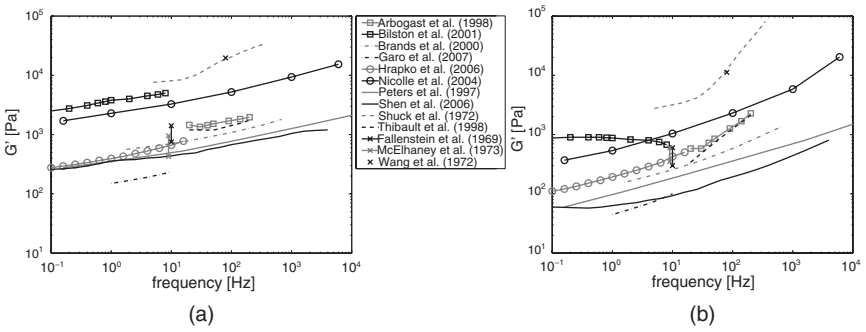

Fig. 12.6 Summary of the linear viscoelastic properties of brain tissue reported in literature

testing methods and protocols used, which makes a comparison of results difficult. Several authors have presented an overview of available literature on the constitutive properties of brain tissue (Ommaya, 1968; Goldsmith, 1972; Thibault and Gennarelli, 1985; Donnelly, 1998; Hrapko et al., 2007b). An overview of the methods and the conditions of materials tested in previous studies is given in Table 12.1. The studies were divided into groups depending on the type of experiment and are summarised in Tables 12.3 and 12.4 in the Appendix. In addition to these studies, some investigators have used techniques like magnetic resonance elastography (Kruse et al., 1999; Manduca et al., 2001, 2003; McCracken et al., 2005; Hamhaber et al., 2007) and ultrasound (Etoh et al., 1994; Lin et al., 1997; Lin and Grimm, 1998; Lippert et al., 2003, 2004), however these are not included in the tables.

12.3.1 Species

Animal brains are often used as a substitute for human brains. The main reasons are that animal brains are easily available and that the *post-mortem* time can be minimised. Human brains have been obtained from autopsies or lobotomies on epileptic patients. In the study of Prange and Margulies (2002), human and porcine brain samples subjected to shear stress relaxation tests were compared. The human samples were on average 29% stiffer than the porcine samples. Takhounts et al. (2003) found human brain tissue samples to be 40% stiffer than bovine samples in stress relaxation experiments. On the other hand, Nicolle et al. (2004, 2005) concluded from dynamic frequency sweep tests that the storage modulus of porcine brain tissue was 17% higher than that of human brain tissue, whereas the loss modulus was similar in both materials. The differences between human and animal brains are often considered relatively small (compared to the effects of other aspects) which enables animal brains to be used as a good substitute for human brains.

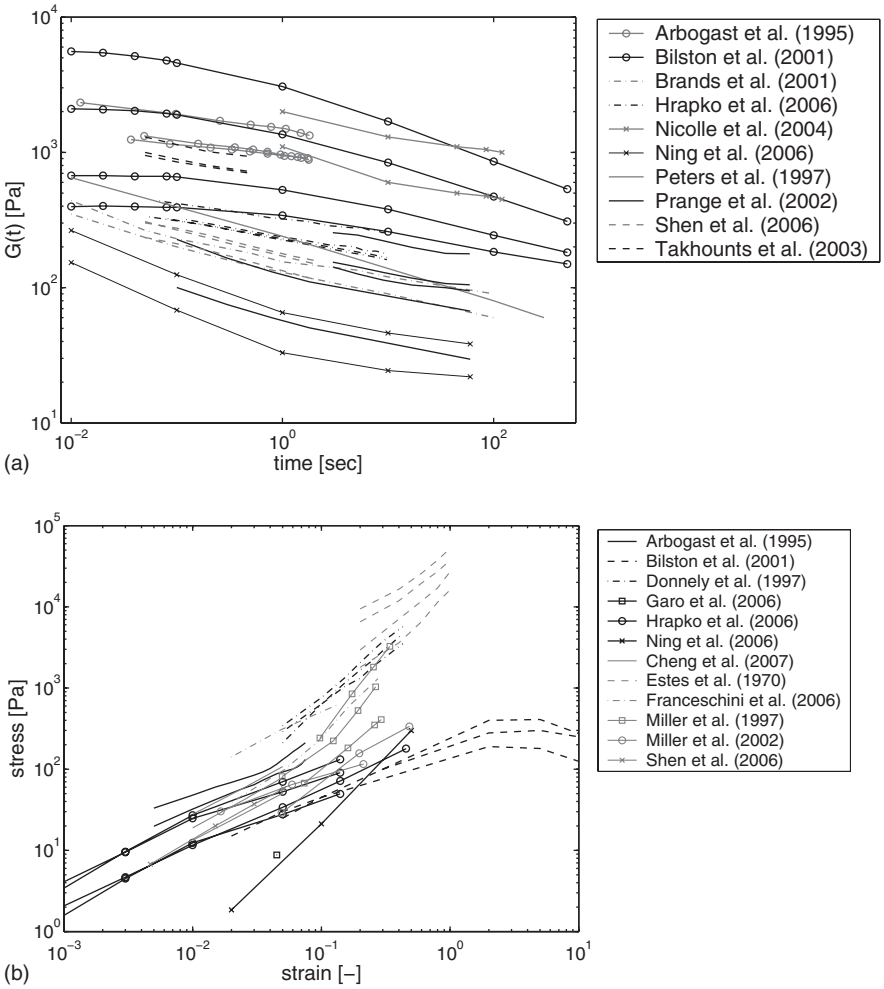

Fig. 12.7 (**a**) Summary of shear stress relaxation experiment results reported in literature. (**b**) Summary of constant strain rate experiment results reported in literature, for shear (*black*) and uniaxial (*grey*) deformation

12.3.2 Boundary Conditions

Besides differences in the material tested, also the test conditions give rise to differences in the results. The method used to attach the sample to the plates of a setup for shear deformation can have a role in the variation in results. Some authors (Darvish and Crandall, 2001; Donnelly and Medige, 1997; Nicolle et al., 2004, 2005; Takhounts et al., 1999, 2003) have used glue to attach samples to the plates, whereas others (Arbogast and Margulies, 1997, 1998; Arbogast et al., 1995; Bilston et al., 1997, 2001; Brands et al., 1999, 2000, 2004; Hrapko

Table 12.1 Overview of previous studies on the mechanical properties of brain tissue

	donor	state	region	Load	test
Dogson (1962)	RMB	vtr	–	C	Cr
Koeneman (1966)	RB, RMB, PB	vtr	Cb	C	DE, Cr
Ommaya (1968)	MB, CB	vtr	CGM	C	PI
Fallenstein et al. (1969)	HB, MB	vv, vtr	Cb	S, C	DE, PI
Estes and McElhaney (1970)	HB, MB	vtr	CR	C	CSR
Galford and McElhaney (1970)	HB, MB	vtr	Cb	C	DE, SR, Cr
Metz et al. (1970)	MB	vv, vtr	CGM	C	ECE
Shuck and Advani (1972)	HB	vtr	CR, T	S	DE
Wang and Wineman (1972)	MB	vv, vtr	CGM	C	DE, PI
McElhaney et al. (1973)	HB, MB	vv, vtr	Cb, CGM	S, C	DE, CSR, PI
Arbogast et al. (1995, 1997); Arbogast and Margulies (1997, 1998)	PB	vtr	Bs, Cb, T, CR	S	DE, SR, CSR
Thibault and Margulies (1996, 1998)	PB	vtr	Cb	S	DE
Bilston et al. (1997, 2001)	BB	vtr	CC	S	DE, SR, CSR
Donnelly and Medige (1997)	HB	vtr	CC, Mb	S	CSR
Miller (1997); Miller et al. (2000); Miller and Chinzei (2002)	PB	vv, vtr	Cb, CGM	C, T	CSR, PI
Peters et al. (1997)	BB	vtr	CR, Mb	S	DE, SR
Prange et al. (1998a, 2000); Prange and Margulies (1999, 2002)	PB, HB	vtr	CR, CC, T	S, C	SR
Brands et al. (1999, 2000, 2004)	PB	vtr	T	S	DE, SR
Takhounts et al. (1999, 2003)	BB, HB	vtr	Cb	S	SR
Darvish and Crandall (2001)	BB	vtr	CR	S	DE
Gefen et al. (2003); Gefen and Margulies (2004)	PB, RMB	vv, vtr	CGM	C	PI
Nicolle et al. (2004, 2005)	PB, HB	vtr	CR,T	S	DE, SR
Franceschini et al. (2006)	HB	vtr	Cb, CC, T	T, C	CSR, Cr
Hrapko et al. (2006)	PB	vtr	CC	S	DE, SR, CSR
Ning et al. (2006)	PB	vtr	Bs	S	CSR, SR
Shen et al. (2006)	PB	vtr	Cb	S, C	DE, SR, CSR
Cheng and Bilston (2007)	BB	vtr	CR	C	SR, CSR
Garo et al. (2007)	PB	vtr	T	S	DE, CSR

Donor: PB – porcine, BB – bovine, HB – human, RB – rabbit, RMB – rat or mouse, MB – monkey, CB – cat. State: vv – in-vivo, vtr – in-vitro. Loading condition: C – compression, S – shear, T – tension. Type of test: DE – dynamic experiment (strain/frequency sweep), SR – stress relaxation test, CSR – constant strain rate test, PI – probe indentation, Cr – creep, ECE – elastic cylinder expansion. Brain region: Cb – cerebrum (white and grey), CC – corpus callosum (white), CR – corona radiata (white), T – thalamus (grey), Mb – midbrain (grey), Bs – brainstem (grey), CGM – cortical grey matter.

et al., 2006; Garo et al., 2007; Prange and Margulies, 1999, 2002; Prange et al., 1998a, 2000; Thibault and Margulies, 1996, 1998; Shen et al., 2006) have used a roughened surface (glass or sandpaper) to avoid slip. Arbogast et al. (1995) and Brands et al. (2000) have found no difference in results when either sandpaper or glue was used to fix the sample to the plates. On the other hand, Nicolle et al. (2004, 2005) have found the dynamic modulus to be dependent on the sample thickness when the samples were not fixed, whereas no variation was achieved when samples were glued to the plates.

When a roughened surface is used, the sample must be uniaxially loaded prior to the shear test which may affect the results of the shear measurements. This can be avoided by gluing the sample to the plates. However, in that case the thickness of the glue layer is unknown and therefore the real height of the sample is unknown. As a consequence, also in case of glue the sample is often uniaxially loaded prior to shear measurements.

Hrapko et al. (2007b) hypothesised that the compression force which has to be imposed to the sample prior to shear tests, affects the measured mechanical properties obtained for the material. To support the hypothesis, shear measurements were conducted with varying amounts of compression for each sample. The amount of pre-compression was found to significantly affect the mechanical properties obtained in shear measurements. Although a sufficient amount of pre-compression is required to prevent the occurrence of slip in subsequent shear measurements, an increasing amount of compression force will yield the deformation state to be a combination of shear and compression rather than pure shear. Furthermore, the friction created between the sample and the plate will lead to a non-homogeneous state with an enlarged cross-sectional area in the middle plane of the sample. The dependence of the shear properties obtained from the measurement on the amount of pre-compression results from a combination of these effects. In Hrapko et al. (2007b), a 20% increase of the shear modulus observed was found when increasing the pre-compression force from 5 to 10 mN. Contrary, Nicolle et al. (2005) have found a 24% decrease of shear modulus in the linear range with pre-compression increasing from 1 to 3%.

12.3.3 Temperature

Although the effect of temperature is an important issue, only a few studies on this topic have been published in the literature (Peters et al., 1997; Brands et al., 2000; Shen et al., 2006; Hrapko et al., 2007b). Particularly important is the difference between room temperature (approximately 23°C) and body temperature (approximately 37°C), to be able to scale results obtained at those different conditions. In addition, a temperature-dependence can be used to extend the frequency/time range of measured data by applying time-temperature superposition.

In Hrapko et al. (2007b), samples from the posterior side of the corona radiata were measured at 37, 30, 23, 15, and 7°C. Dynamic frequency sweep tests and stress relaxation tests were conducted to obtain data in the linear and large strain regime, respectively. To characterise the temperature dependence of brain tissue, time-temperature superposition (TTS) was applied. This method was previously used for brain tissue by Peters et al. (1997), Brands et al. (2000), and by Shen et al. (2006). For different temperatures, sets of isothermal characteristics were obtained within an equal frequency/time range. These characteristics were shifted along the frequency/time axis, to form a master curve. The reference temperature T_0 was chosen to be 37°C. The horizontal shift depends only on the difference between the reference temperature and the temperature of the shifted characteristic and can be described by the horizontal shift factor $a_T(T,T_0)$ (Ferry, 1980). Besides the horizontal shift factor, also a vertical shift, characterised by the shift factor $b_T(T,T_0)$ has to be applied. First, the horizontal shift factor a_T is determined from phase angle δ, only, to satisfy:

$$\delta(\omega, T) = \delta(a_T\omega, T_0). \tag{12.10}$$

After applying this shift to the dynamic modulus G^*, the vertical shift factor b_T is determined to satisfy:

$$G^*(\omega, T) = \frac{1}{b_T} G^*(a_T\omega, T_0). \tag{12.11}$$

In the case of the stress relaxation experiments, the shift factors are determined to satisfy:

$$G(t, T) = \frac{1}{b_T} G(\frac{t}{a_T}, T_0). \tag{12.12}$$

Hrapko et al. (2007b) found the dynamic modulus and the phase angle to be clearly temperature dependent with a horizontal shift factor a_T between 23 and 37°C of up to 11, whereas the vertical shift factor b_T was close to one.

In most studies (Peters et al., 1997; Brands et al., 2000; Shen et al., 2006; see Table 12.2), mechanical test results of brain tissue have been found to be clearly

Table 12.2 Shift factors a_T and b_T for scaling results of dynamic frequency sweep (DFS) and stress relaxation (SR) tests from 23 to 37°C

	Type of test	Mean	
		a_T	b_T
Peters et al. (1997)	DFS, SR	6.7	2.6
Brands et al. (2000)	DFS	5.5	1.03
Shen et al. (2006)	DFS	~5	1
Hrapko et al. (2007b)	DFS, SR	8.5–11	1.3–0.99

temperature dependent and can be scaled by a horizontal shift factor a_T and a negligible vertical shift factor b_T. In one study (Arbogast and Margulies, 1997) the results were found to be independent of temperature.

12.3.4 Anisotropy

Some parts of the brain may show anisotropic behaviour because of the underlying microstructure. Whereas grey tissue was found to be nearly isotropic, white matter was found to be anisotropic with different degrees of anisotropy (Prange and Margulies, 2002). Based on its highly organised structure the most anisotropic region is expected to be the corpus callosum (white matter). Arbogast et al. (1995) and Arbogast and Margulies (1998) tested the anisotropy of the brainstem in 2.5% dynamic frequency sweep experiments in three different orientations based on the fibre direction. The differences were found to be up to 30%. Prange et al. (2000) and Prange and Margulies (2002) identified the anisotropy of brain tissue at large strain (up to 50%) shear experiments on white and grey matter samples. White matter behaviour was more anisotropic (31–48% difference), while grey matter was nearly isotropic (12% difference). By studying interregional differences, grey matter from the thalamus was found to be approximately 40 and 12.5% stiffer than white matter from the corpus callosum and the corona radiata, respectively. Nicolle et al. (2004, 2005) investigated anisotropy within the corona radiata (white matter). However, their observations did not allow any conclusion on the anisotropy.

Hrapko et al. (2007b) tested samples from the corona radiata in three planes (sagittal, coronal, transverse) in shear. The variation caused by anisotropy within the plane of testing was highest in the sagittal plane, whereas the smallest variation was found to be in the coronal plane. By comparing the stress relaxation results it was observed that the amount of anisotropy increases with strain, which was also observed by comparing stress relaxation (large strain) with dynamic frequency sweep (small strain) results. Anisotropy of brain tissue can play a role in the variation of results of mechanical tests. In the study by Hrapko et al. (2007b), the average differences between the maximum and minimum found within the testing plane from dynamic frequency sweep and stress relaxation tests are 25–40% and 32–54%, respectively, where the ratios between the results from the coronal/sagittal and transverse/sagittal planes were approximately 1.3.

12.3.5 Post-Mortem Time and Sample Preparation

The tissue and also its mechanical properties may degenerate with increasing *post-mortem* time due to various reasons (e.g. autolytic processes, completion of rigor mortis, osmotic swelling, etc.). This degeneration effect may be

temperature dependent in that the rate of degeneration possibly decreases with decreasing temperatures. Some authors (Shuck and Advani, 1972; Darvish and Crandall, 2001; Donnelly and Medige, 1997; Nicolle et al., 2004, 2005; Peters et al., 1997; Takhounts et al., 1999, 2003) have tested samples several days *post-mortem*, whereas others (Arbogast and Margulies, 1998; Thibault and Margulies, 1998; Brands et al., 2000; Bilston et al., 2001; Prange and Margulies, 2002; Hrapko et al., 2006) within a few hours *post-mortem*. Metz et al. (1970) have reported a 30–70% decrease of the tissue response to the inflation of a balloon catheter, from live to 3/4 hour *post-mortem*. No change of measured properties caused by higher *post-mortem* time was found by McElhaney et al. (1973) up to 15 hours *post-mortem* and by Darvish and Crandall (2001) in 3–16 days. Nicolle et al. (2004, 2005) compared samples measured at 24 and 48 hours *post-mortem* and found only a 6% increase of dynamic modulus. Shen et al. (2006) examined samples up to 7 days *post-mortem*, and reported only a small variation of the material properties.

Garo et al. (2007) found the onset of stiffening of the shear modulus by approximately 27 Pa/h to be after 6 hours *post-mortem*. They applied a series of loading-unloading cycles at a constant shear rate of $1\ s^{-1}$ to the samples in two different test sequences. The purpose of these tests was to determine the presence of any potential evolution of mechanical properties due to either increasing *post-mortem* time or mechanical history of the sample. The test sequences consisted of loading-unloading cycles separated by a recovery period of 10 minutes and differed in the shear strain amplitude of the loading-unloading cycles, see Fig. 12.8(a) and (b).

Three different test protocols have been used in this study, consisting of different combinations of sample preparation method and test sequence (protocol 1: microtome + sequence A; protocol 2: meat slicer + sequence A; protocol 3: meat slicer + sequence B). The aim of the first two test protocols was to investigate a possible effect of the cutting procedure on the mechanical response of the samples. The preparation procedure could potentially affect the mechanical behaviour of the material due to the mechanical history applied during this procedure. The third test protocol was applied with the goal to

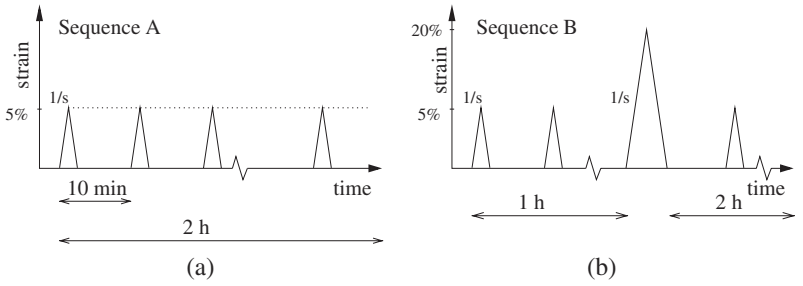

Fig. 12.8 Different sequences of transient tests. Reprinted from Garo et al. (2007), with permission from IOS Press

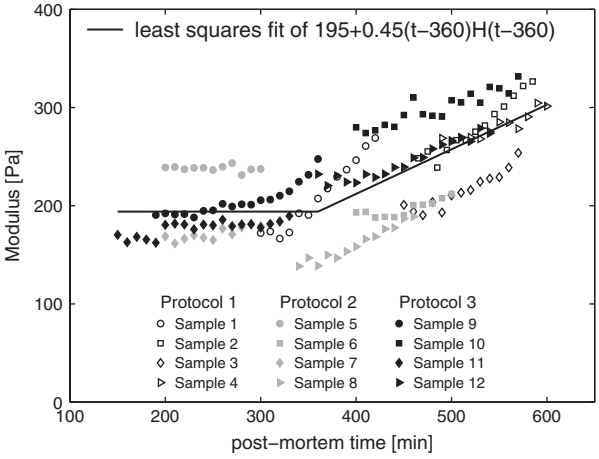

Fig. 12.9 Modulus at 4.5% strain versus post-mortem time. Reprinted from Garo et al. (2007), with permission from IOS Press

investigate the effect of a well-defined mechanical history on the mechanical response of the tissue at a relatively long time scale. Figure 12.9 shows the instantaneous modulus calculated from the shear stress level at 4.5% strain versus the *post-mortem* time for all three protocols. The mechanical response of brain tissue was found to stiffen with increasing *post-mortem* time, if this *post-mortem* time exceeds a threshold value of 6 hours. The onset of changes in the mechanical behaviour with increasing *post-mortem* time appeared to depend on the mechanical history of the tissue. Furthermore, this mechanical history may be affected by the sample preparation procedure. Mechanical loading during sample preparation can accelerate the evolution of mechanical properties with advancing *post-mortem* time. Moreover, for reproducible results, brain tissue should generally be tested within a *post-mortem* time of 6 hours. For studies in which the tissue is tested at relatively longer *post-mortem* times, the mechanical properties obtained can be expected to be relatively stiff.

Those authors (Arbogast and Margulies, 1998; Thibault and Margulies, 1998; Brands et al., 2000; Bilston et al., 2001; Prange and Margulies, 2002) who measured their results on shorter *post-mortem* times argued that this minimises deviations caused by higher *post-mortem* times. However, also several studies were made with larger *post-mortem* times.

12.3.6 Compression

The mechanical behaviour of brain tissue has been tested mostly in-vitro in shear (Arbogast and Margulies, 1998; Bilston et al., 2001; Brands et al., 2000; Garo et al., 2007; Ning et al., 2006; Peters et al., 1997; Prange and Margulies, 2002; Shen et al., 2006; Shuck and Advani, 1972; Thibault and Margulies,

1998), but also in compression (Cheng and Bilston, 2007; Estes and McElhaney, 1970; Miller, 1997, 2005; Prange and Margulies, 2002; Shen et al., 2006) and tension in some studies (Franceschini et al., 2006; Miller and Chinzei, 2002; Velardi et al., 2006). The effect of certain conditions of compression measurements has been evaluated as well.

Miller (1997), Shen et al. (2006) and Hrapko et al. (2007a) highlighted the tendency of a sample to adhere to the top plate of a rheometer, which takes place even before touching the actual sample, leading to a tensile loading of the sample. This is caused by the surface tension of a thin fluid layer on top of the sample, and may affect the measurement results. Whereas Miller (1997) and Estes and McElhaney (1970) have started their compression tests without initial contact between the sample and the top plate, Shen et al. (2006) and Cheng and Bilston (2007) conducted compression tests with the top plate initially touching the sample. Measurements according to these two methods were used in Hrapko et al. (2007a) to examine the effect of a fluid layer on the measured response. When starting the compression without initial contact between the sample and the top plate, the initial compressive response of the sample was underestimated up to 6.6 times compared to the response to compression with initial contact. A similar difference between the stress response of these two protocols was obtained from a model prediction as well. It was shown, that the difference was caused by the state of the material being different in the beginning of the loading part of each sequence. Due to the tensile loading before a compression test without initial contact, the material is not in an equilibrated state at the onset of the compression test, which leads to a more compliant initial response. Therefore, it was concluded that a compression test should start with the top plate touching the sample, after the sample is fully recovered.

There are a few studies discussing the effect of friction between the sample and the loading plate. Wu et al. (2004) has used data and an Ogden hyper-elastic model from Miller (1997) in an FE model. They have compared results for friction coefficients of 0–0.5, for different strain rates, and also different specimen aspect ratios (diameter/height). They have concluded that a higher friction coefficient will increase the reaction force obtained during compression measurements. Also, the smaller the specimen aspect ratio, the smaller the friction effect on the measured results is. The difference in stress results of 20% strain compression with friction coefficients of 0.3 and 0.1 versus frictionless compression was up to 60 and 10%, respectively. Miller (2005) has investigated the effect of friction coefficients of 0 to 0.1 on the results from FE model predictions. The stress increase for a 20% strain compression due to friction coefficients of 0.1 and 0.05 was up to 15% and 7.5%, respectively. Cheng and Bilston (2007) compared experimental results and model predictions of pure slip, slip with friction of 0.1, 0.3 and 0.5, and a no–slip boundary condition in compression. The peak reaction force of a no–slip boundary condition obtained from numerical simulations was found to be 3 times stiffer than the experimental results. The equilibrium reaction force obtained from measurements with a no–slip boundary condition was found to be 1.64 times higher that those obtained with

a slip boundary condition. Notice, that friction coefficients chosen in these studies are relatively high. Friction is apparently an important aspect which may influence compression measurement results and therefore was examined further in Hrapko et al. (2007a). In that study, the rate-dependence of friction in the interface between brain tissue and a Teflon–coated plate was investigated using shear measurements. This friction was found to be non-Coulombic due to its rate-dependence and the absence of a compression force during the shear measurements. It was concluded that the specimen/plate friction significantly affects the measured results by increasing the stress response. This effect should not be ignored when interpreting compression results.

12.3.7 Consequences for Characterisation of Brain Tissue

All of the aspects discussed can have an effect on the mechanical properties measured. Specifically, the effect of different temperatures and the effect of pre-compression on linear and large strain results were discussed, as well as differences caused by anisotropy. In the combined results of Hrapko et al. (2007b) and Garo et al. (2007), each of these topics was investigated in otherwise comparable conditions.

The considerable differences in mechanical properties as reported in literature may be caused by variations in the material tested, test conditions, testing protocols, and by other reasons. Generally, a combination all of these experimental aspects will determine the mechanical properties measured. Therefore, in studies aimed at determining the mechanical behaviour of soft tissues like brain tissue, the experimental conditions should be carefully controlled and documented. However, notice that the spread in absolute values of comparable results (see Figs. 12.6 and 12.7) from these studies is about two decades, much more than the variation found due to anisotropy, *post-mortem* time, temperature and pre-compression.

12.4 Large Strain Response and Modelling

12.4.1 Large Strain Behaviour of Brain Tissue

Large strain deformation of biological tissue may lead to damage which affects its mechanical behaviour. The occurrence of mechanical damage was investigated by Hrapko et al. (2006) with a series of constant shear rate experiments with increasing strain amplitude. Results from these experiments are presented for two samples in Fig. 12.10. In Fig. 12.10(b), the 0.01 strain limit for linear behaviour is clearly visible in the beginning of each loading part. There was no yield or failure visible for the tested strain range. By comparing the loading parts of different cycles, which were all applied at an identical strain rate, it can be observed that there is no significant immediate mechanical damage affecting

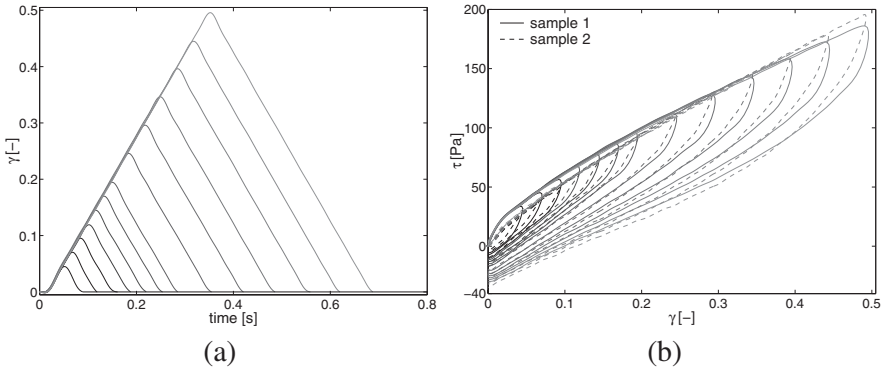

Fig. 12.10 Results of constant shear rate ($1.5\,s^{-1}$) experiments with increasing strain amplitude. (**a**) Applied shear strain, (**b**) stress-strain response. Reprinted from Hrapko et al. (2006), with permission from IOS Press

the stress-strain behaviour due to previous shear deformations (up to strains of 0.45). These results show that mechanical measurements up to a strain of 0.45 are reproducible for short time scales. The conclusions are limited to the time scale of the experiment. It is important to make a clear distinction between functional and mechanical damage. Functional damage can be considered as injury, i.e. change or loss of functionality of the brain tissue, whereas mechanical damage only affects the mechanical properties of the tissue. At these strain levels, functional damage may still occur (as observed by for example Bain and Meaney (2000) and Morrison III et al. (2003)) and at larger time scales also mechanical changes could develop. This observation is in agreement with results of Prange and Margulies (2002) who reported no change in long term modulus and no structural changes in the tissue during stress relaxation experiments up to a strain of 0.5 and shear rates of $8.33\,s^{-1}$. Furthermore, no maximum in the stress-strain response was found even though the samples were tested up to a strain of 0.5, which is in agreement with the findings of Arbogast et al. (1995), Bilston et al. (2001), and Donnelly and Medige (1997).

Constant shear rate experiments at different shear rates show the non-linear strain rate sensitivity of brain tissue, see Fig. 12.11. From these results it can be observed that also in the non-linear range, the stress as a function of strain is strain rate dependent and that the response stiffens with increasing strain rate. In Fig. 12.12 results are shown from stress relaxation experiments. During the loading phase the stress response weakens above the linear viscoelastic strain limit. During stress relaxation, the relaxation modulus does not reach a plateau value within the time range allowed, see Fig. 12.12(b). This is supported by the findings of other researchers who performed stress relaxation experiments (Arbogast et al., 1995; Bilston et al., 2001; Brands et al., 2000; Nicolle et al., 2005; Prange and Margulies, 2002; Takhounts et al., 2003). A decrease of the relaxation modulus with increasing strain was found in the stress relaxation

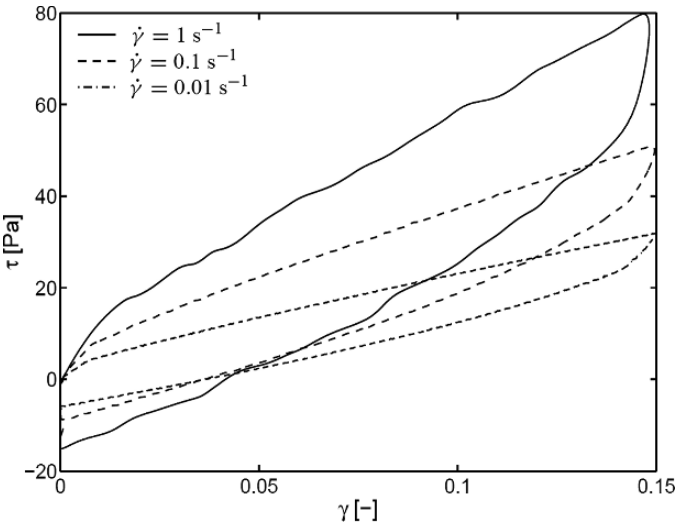

Fig. 12.11 Stress-strain response obtained during constant shear rate experiments at different shear rates. Reprinted from Hrapko et al. (2006), with permission from IOS Press

measurements, which is in agreement with Arbogast et al. (1995), Bilston et al. (2001), Brands et al. (2000), Nicolle et al. (2005), Peters et al. (1997) and Prange and Margulies (2002). This effect was decreasing with strain level for strains higher than the linear viscoelastic limit which differs from the results of Brands et al. (2000) and Nicolle et al. (2005). The difference may be attributed to the non-homogeneous shear field in case of a conventional centred rotational shear setup. In Hrapko et al. (2006) an approximately homogeneous shear field was obtained by placing the samples at the edge of the plate. Similar behaviour was

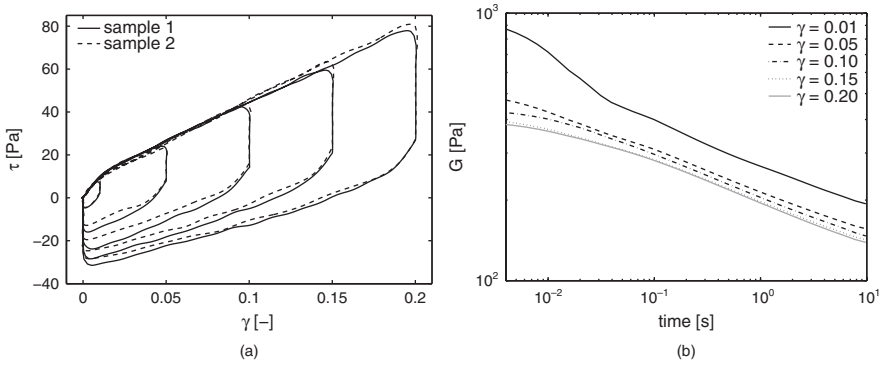

Fig. 12.12 Results of stress relaxation experiments in shear. (**a**) stress-strain behaviour; **(b)** stress relaxation modulus. Reprinted from Hrapko et al. (2006), with permission from IOS Press

observed in simple shear measurements on a translational shearing device by Arbogast et al. (1995), Prange and Margulies (2002), and Takhounts et al. (2003). However, although less pronounced, the same effect was also observed by Bilston et al. (2001) with a centred rotational configuration.

12.4.2 Volumetric Behaviour

Most studies have focused on the resistance to deviatoric deformations of brain tissue. In most cases the hydrostatic response is assumed to be purely elastic with a bulk modulus that is several orders of magnitude higher than the shear modulus. The bulk modulus reported for brain tissue ranges from 2.1 GPa (McElhaney et al., 1976) to 2.5 GPa, based on the velocity of dilatational waves in brain tissue which has been measured in ultrasonic experiments and was found to be approximately 1550 m/s (Etoh et al., 1994; Goldman and Hueter, 1956; Lin et al., 1997).

12.4.3 Constitutive Modelling

A number of constitutive models have been developed to describe the mechanical behaviour of brain tissue. Many researchers used an integral viscoelastic model, often in combination with Ogden hyper-elasticity to describe the viscoelastic behaviour of brain tissue (Darvish and Crandall, 2001; Mendis et al., 1995; Miller, 1999; Nicolle et al., 2005; Prange and Margulies, 2002; Takhounts et al., 2003), and some propose a differential constitutive equation (Bilston et al., 2001; Brands et al., 2004; Donnelly and Medige, 1997). The ability of a constitutive model to describe the complex mechanical response is important for reliable simulations of head injury. Therefore a model is required that is able to describe the response to large deformations in different deformation modes. The ability of a model to describe the anisotropic behaviour of brain tissue may be important for some regions of the brain, although the degree of anisotropy observed for brain tissue is relatively low, see Section 3.4 (Nicolle et al., 2005; Prange and Margulies, 2002; Velardi et al., 2006; Hrapko et al., 2007b).

Arbogast et al. (1995) fitted an integral viscoelastic model with a third order polynomial for the instantaneous elastic response to data from stress relaxation tests. Bilston et al. (2001) concluded that a quasi linear viscoelastic (QLV) integral model is not sufficient and used a multi mode upper convected Maxwell model in combination with a Mooney-Rivlin hyperelastic response multiplied by a strain dependent function. This model worked well, comparing results from stress relaxation, constant strain rate and dynamic measurements in shear. However it was not validated in other deformation modes. Two weaknesses of this non-linear constitutive equation were mentioned, that is computational expensiveness and complexity. Brands et al. (2004) proposed a non-linear

viscoelastic constitutive model in a differential framework in combination with a second order Mooney-Rivlin model. Because of a lack of results from compression tests they were unable to specify all parameters. However, because of the higher order terms present in this model, application for strains outside the range for which the model was characterised should be done with care. Darvish et al. (1998) and Darvish and Crandall (1999, 2001) investigated the use of two integral constitutive models, that is the QLV model and a third order Green-Rivlin viscoelastic nonlinear model (GR). The latter model was shown to be superior at frequencies above 44 Hz. The elastic response of the QLV model showed strain hardening for shear strains above 10% which is in contradiction with QLV models proposed by Prange et al. (1998b) and Mendis et al. (1995). Also Takhounts et al. (1999, 2003) used linear, quasi linear and GR nonlinear viscoelastic constitutive models that were fitted to results from stress relaxation experiments. Donnelly and Medige (1997) used a nonlinear viscoelastic solid model to fit data from constant strain rate experiments at high shear rates (30–90 s^{-1}). Mendis et al. (1995) proposed a QLV constitutive equation with a Mooney-Rivlin elastic function, where the viscoelastic properties were characterised by time dependent coefficients. They fitted this constitutive equation to the measured data from constant strain rate compression tests published by Estes and McElhaney (1970). Miller (1997) presented a QLV model with a Mooney-Rivlin function and fitted it to the results from unconfined compression experiments on porcine brain tissue. The model agreed well with the experimental data for compression levels reaching 30%. Miller (1999) fitted a linear viscoelastic model with a polynomial strain energy function to the same results and obtained similar results as with the previous model. Miller (2000) modified the non-linear model presented in Miller (1997) and then compared the prediction with the coefficients from in-vitro tests with results from in-vivo indentation experiments. Predicted forces were approximately 31% lower than those recorded during the experiment. Based on the results from tensile experiments Miller (2001) proposed a nonlinear viscoelastic model based on the Ogden strain energy hyperelastic function. The model was found to apply well for tissue properties in compression as well as in tension for strains up to 30%. Nicolle et al. (2004) fitted a five mode viscoelastic model in a QLV framework to dynamic frequency data, where an Ogden hyperelastic function was used to describe the equilibrium moduli from relaxation tests at different strain levels. In Prange et al. (1998b, 2000) and Prange and Margulies (2002), the non-linear material properties were modelled with a QLV model in combination with a first order Ogden hyperelastic function to include energy dissipation. This model predicted data from shear measurements well and the result was validated with compression data.

In summary, several authors (Darvish and Crandall, 2001; Mendis et al., 1995; Miller, 1999; Nicolle et al., 2005; Prange and Margulies, 2002; Takhounts et al., 2003) have used an Ogden model in a viscoelastic framework to describe the non-linear response of brain tissue. In this model, the elastic strain energy is given by:

$$W = \frac{\mu}{\alpha}(\lambda_1^\alpha + \lambda_2^\alpha + \lambda_3^\alpha - 3), \tag{12.13}$$

where λ_i are the principal stretch ratios. For incompressible materials, this leads to the following elastic stress-strain relation:

$$\boldsymbol{\sigma} = \mu(\lambda_1^\alpha \vec{n}_1\vec{n}_1 + \lambda_2^\alpha \vec{n}_2\vec{n}_2 + \lambda_3^\alpha \vec{n}_3\vec{n}_3), \tag{12.14}$$

with $\vec{n}_i$ the principal stretch directions. Note that an additional hydrostatic term should be added to this stress tensor. Other authors (e.g. Mendis et al., 1995; Miller, 1997; Bilston et al., 2001; Brands et al., 2004; Hrapko et al., 2006) have used a Mooney-Rivlin model instead:

$$W = \frac{C_1}{2}(I_1 - 3) + \frac{C_2}{2}(I_2 - 3) \quad \text{with} \quad \boldsymbol{\sigma} = C_1\boldsymbol{B} - C_2\boldsymbol{B}^{-1}, \tag{12.15}$$

with $\boldsymbol{B}$ the left Cauchy-Green strain tensor and I_1 and I_2 its invariants. In some cases, additional higher order terms or an additional non-linear function have been used (Bilston et al., 2001; Brands et al., 2004; Hrapko et al., 2006). In most studies (Arbogast et al., 1995; Darvish and Crandall, 2001; Mendis et al., 1995; Miller, 2001; Nicolle et al., 2004; Prange and Margulies, 2002; Takhounts et al., 2003) an integral constitutive equation was used to model viscoelastic effects in the mechanical behaviour of brain tissue. However, Bilston et al. (2001), Darvish and Crandall (1999, 2001), and Takhounts et al. (2003) have pointed out the incapability of the QLV integral models to describe the large strain response of this material. The differential formulation provides a general framework for describing viscoelastic material behaviour with the appropriate choice of the (linear or non-linear) constitutive relations for the elastic and viscous components. The differential framework was used by Bilston et al. (2001), Brands et al. (2004), Donnelly and Medige (1997) and Hrapko et al. (2006). In the model by Hrapko et al. (2006), a non-linear elastic mode is used in combination with a number of viscoelastic modes showing viscous shear thinning behaviour. The model is formulated in a general differential viscoelastic framework that is appropriate for large deformations (Peters and Baaijens, 1997). The most characteristic aspect of this model is however the non-linear equilibrium response which is given by:

$$\boldsymbol{\sigma} = G_e[(1-A)\exp(-C\sqrt{bI_1 + (1-b)I_2 - 3}) + A][b\boldsymbol{B}^{\mathrm{d}} - (1-b)(\boldsymbol{B}^{-1})^{\mathrm{d}}]. \tag{12.16}$$

Constitutive models for brain tissue should be validated in different deformation modes such as shear and compression. Some studies have presented combined shear and compression measurement results and subsequent model predictions (Prange and Margulies, 2002; Shen et al., 2006; Hrapko et al., 2007a). Prange and Margulies (2002) have shown a good model prediction of

shear stress relaxation; however the compressive response was validated only with the equilibrium stress obtained from compression stress relaxation. On the other hand, Shen et al. (2006) have validated a simplified version of a constitutive model developed by Bilston et al. (2001) with constant strain rate measurements in compression only up to a strain of 5%. However, in both of these studies, the model tends to underpredict the tissue response in compression deformation, which is similar to results obtained by Hrapko et al. (2007a), where compression and shear results obtained for the same sample on the same setup were used.

12.5 Conclusions

Studies on the characterisation of brain properties by different laboratories using different testing protocols have yielded a wide range of results. Concerning the results of the dynamic frequency sweep tests, all the samples show the same behaviour: the storage and loss moduli increase with frequency, however, the results are orders of magnitude different. Mutually similar results were found by Peters et al. (1997), Brands et al. (1999), Hrapko et al. (2006, 2007b), and Shen et al. (2006). However, stiffer properties were obtained by Arbogast and Margulies (1998), Thibault and Margulies (1998), Bilston et al. (2001), and Nicolle et al. (2004). The differences between various studies cannot be attributed entirely to species, regional variation or to anisotropy. Among other factors, a possible source for differences in reported properties may be the preparation techniques used, the difference in *post-mortem* times, temperature and boundary conditions of the test. However, despite all these difficulties in assessing the mechanical properties, it seems that most recent studies indicate that the storage shear modulus of brain tissue is at least in the range of 100–1000 Pa for a frequency of 10 Hz.

The importance of including the non-linearity of the mechanical response that is experimentally observed in constitutive models that are used for head model simulations should be evaluated. At least for the linear viscoelastic properties of brain tissue, a universally accepted data set is required for the injury biomechanics community. Only then, numerical head models may be accepted as injury predictors and can be incorporated in safety regulations.

Appendix: Studies Characterising the Mechanical Properties of Brain Tissue

The data available in literature were divided into groups depending on the type of experiment. An overview of measurements in shear deformation is given in Table 12.3, whereas Table 12.4 includes measurements in uniaxial deformation.

Table 12.3 Overview of experimental studies on brain tissue in shear

	Dynamic tests donor age	*post-mortem* time [h]	attach. method	T [°C]	frequency [Hz]	strain [%]
Fallenstein et al. (1969)	fg	2.5–62	g, rs	37	9–10	7–24.5
Shuck and Advani (1972)	fg	–	–	37	5–350	1.23
McElhaney et al. (1973)	fg	2.5–15	no	37	9–10	–
Arbogast et al. (1995); Arbogast and Margulies (1997, 1998)	p, fg	4	no, g, rs	5–25	20–200	2.5, 5, 7.5
Thibault and Margulies (1996, 1998)	p, fg	3	no	~25	20–200	2.5, 5
Bilston et al. (1997, 2001)	–	8	sp	37	0.01–20	0.015–2
Peters et al. (1997)	nfg	27–51	–	7–37	0.016–16	0.2–5
Brands et al. (1999, 2000)	nfg	4	sp, no, g	4–38	0.1–16	0.1–10
Darvish and Crandall (2001)	nfg	72–288	g	37	0.5–200	1–20
Nicolle et al. (2004, 2005)	nfg	24–48	g	37	0.1–9000	0.01–10
Hrapko et al. (2006)	nfg	5	sp	37	0.04–16	1
Shen et al. (2006)	fg	48–120	sp	10–37	0.016–16	0.04–400
Garo et al. (2007)	nfg	2–10	sp	37	1–10	1
	Stress relaxation tests **donor age**	***post-mortem* time [h]**	**attach. method**	**T [°C]**	**strain [%]**	**relax. time [s]**
Arbogast et al. (1995)	p	4	no	~25	2.5, 5, 7.5	1.8
Bilston et al. (1997, 2001)	–	8	sp	37	0.001–15	3000
Peters et al. (1997)	nfg	27–51	–	7–37	1	300
Prange et al. (1998a, 2000); Prange and Margulies (1999, 2002)	p, nfg, fg	3–5	no	~25	2.5–50	60
Takhounts et al. (1999, 2003)	fg	48	g	~25	12.5–50	0.5
Brands et al. (2000)	nfg	4	sp	38	5–20	100
Nicolle et al. (2004, 2005)	nfg	24–48	g	37	0.1–50	300
Hrapko et al. (2006)	nfg	5	sp	37	1–20	10

Table 12.3 (continued)

Ning et al. (2006)	p	5	no	–	2.5–50	60
Shen et al. (2006)	fg	48–120	sp	10–37	0.5, 5, 10, 20	2
	Constant strain rate tests					
	donor age	*post-mortem* time [h]	attach. method	T [°C]	strain rate [s^{-1}]	strain [%]
Arbogast et al. (1995)	p	4	no	~25	–	8
Donnelly and Medige (1997)	fg	72–96	g	~25	30, 60, 90, 120, 180	0.28–12.5
Bilston et al. (2001)	–	8	sp	37	0.055, 0.2335, 0.947	2000
Hrapko et al. (2006)	nfg	5	sp	37	1, 1.5	1–50
Ning et al. (2006)	p	5	no	–	20–25	50
Garo et al. (2007)	nfg	2–10	sp	37	1	5

Donor age: p – paediatric, nfg – not full grown, fg – full grown. Attachment method: no – no glue, g – glue, sp – sandpaper, rs – roughened surface. T – test temperature.

Table 12.4 Overview of experimental studies of brain tissue in uniaxial deformation

	Dynamic tests					
	donor age	*post-mortem* time [h]	attach. method	T [°C]	Strain [–]	frequency [Hz]
Koeneman (1966)	–	0.5–3	no	22	$\sim 10^{-5}$	80–350
Galford and McElhaney (1970)	fg	1–12	no	37	–	31, 34
McElhaney et al. (1973)	fg	2.5–15	no	37	–	31, 34
	Stress relaxation tests – Compression					
	donor age	*post–mortem* time [h]	attach. method	T [°C]	Strain [–]	relax. time [s]
Galford and McElhaney (1970)	fg	1–12	no	37	–	80
Prange et al. (2000)	fg	5	no	~25	–0.05, –0.3, –0.5	60
Cheng and Bilston (2007)	nfg	–	no, g	–	–0.05	3500
	Constant strain rate tests					
	donor age	*post-mortem* time [h]	attach. method	T [°C]	Strain [–]	strain rate [s^{-1}]
	Compression					
Estes and McElhaney (1970)	fg	1–12	no	37	–1	0.08, 0.8, 8, 40
McElhaney et al. (1973)	fg	2.5–15	no	37	–1.2	0.1, 1, 10, 65
Miller (1997)	nfg	–	no	~22	–0.34	$64x10^{-2}$–$64x10^{-7}$
Franceschini et al. (2006)	fg	–	g	37	–0.26	5.5–9.3
Cheng and Bilston (2007)	nfg	–	no, g	–	–0.05	10^{-2}–10^{-4}
	Tension					
Miller and Chinzei (2002)	nfg	–	g	~22	0.48	$64x10^{-2}$–$64x10^{-4}$
Franceschini et al. (2006)	fg	–	g	37	1.335	5.5–9.3
	Creep tests – Compression					
	donor age	*post-mortem* time [h]	attach. method	T [°C]	Load [N, Pa]	time [s]
Dogson (1962)	–	–	–	16–26	0.2 N	15–90000
Koeneman (1966)	–	0.5–3	–	22	–	0.2–200
Galford and McElhaney (1970)	fg	1–12	no	37	3447, 6895 Pa	1000
Franceschini et al. (2006)	fg	–	no	~25	2–12 N	54000

Donor age: p – paediatric, nfg – not full grown, fg – full grown. Attachment method: no – no glue, g – glue, sp – sandpaper, rs – roughened surface. T – test temperature.

In general, dynamic tests were done by applying an oscillatory sinusoidal strain with a certain amplitude with or without varying the strain/frequency. Note that the linear viscoelastic properties should be determined from dynamic tests in the linear regime, which was found to be limited to 1% shear strain by Brands et al. (1999) and Nicolle et al. (2004). Dynamic tests include also free vibration experiments. Constant strain rate tests consist of loading the sample by keeping a constant strain rate up to a certain strain level. In stress relaxation tests, the subsequent relaxation of the mechanical response is measured. Creep tests were done by applying a certain load on the sample and recording the strain response.

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Chapter 13
Role of Falx on Brain Stress-Strain Responses

Narayan Yoganandan, Jianrong Li, Jiangyue Zhang and Frank A. Pintar

Abstract The objective of this chapter is to provide a review of the role of falx cerebri on brain mechanics, specifically stress and strain responses due to dynamic loading. Because stress-strain responses are inherently intrinsic, the review is focused on physical and computational models using the finite element method. In order to maintain the focus, although experimental animal models are used as validations tools for ensuring the confidence in the finite element or physical model output, discussions from biological tests are not a subject matter. While finite element modeling of the human head has been a subject matter of investigation for decades, a review of literature provides very few analyses regarding the role of falx on the internal stress-strain responses of the brain. As described, physical and finite element models have shown that the falx cerebri, present in the human head, affects the intrinsic response of the brain under contact- and inertia-induced dynamic loads. Physical models using a brain substitute have also shown a similar response. Regional stresses and strains from these models are discussed. The chapter concludes with some recommendations for further studies.

Keywords Acceleration · brain trauma · finite element model · strain

13.1 Introduction

The falx cerebri is an important soft tissue structural component in the human brain, considered as the region that differentiates the left and right hemispheres. Its deformability characteristics allow motion, and hence, transfer of the external insult to the other components of the brain under mechanical loads. The role of this component forms the focus of this chapter. While it is logical to describe its biological, anatomical, and physiological aspects, to stay within

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the mechanics focus, the reader is referred to literature. From external mechanical loading perspective, rotational and/or translational accelerations due to incidents such as fall-, motor vehicle- and athletic-related events are attributed to be responsible based on clinical, epidemiological, and biomechanical studies (Abel et al., 1978; Adams et al., 1982, 1983, 1984; Gennarelli and Meaney, 1996; Gennarelli and Thibault, 1982; Graham et al., 1993; Higgens and Unterharnscheidt, 1969; Hodgson et al., 1983; Holbourn, 1943; Imajo et al., 1987; Imajo and Kazee, 1992; Margulies and Thibault, 1992; McLean, 1995; Morris et al., 1993; Ommaya and Gennarelli, 1974; Peerless and Rewcastle, 1967; Pintar et al., 2000; Sosin et al., 1995; Thibault and Gennarelli, 1985). It is therefore pertinent to examine the role of falx under these loading conditions. A review of biomechanical literature shows that physical and computational models have been used to describe its role on the intrinsic responses of the human brain due to dynamic loading. Consequently, these studies are briefly reviewed in this chapter.

13.2 The Role of Falx Using Physical Models Under Angular Acceleration

During an investigation of severe diffuse axonal injuries secondary to inertial loading, i.e., angular accelerations without direct head impact, experiments were conducted using physical skull-brain models of the baboon and human. The anterior portion of the baboon or human skull was separated from its posterior half by sectioning coronally. The anterior half was potted into an aluminum cylinder, fixed to the impulse loading device such that the coronal plane could be viewed. Covering the foramen magnum, the model simulated the brain using an optically transparent material (Sylgard Medical gel, Q7-2218, Dow Corning Inc., Midland, USA) mixed in equal ratio of the polymer to catalyst. The falx was modeled using polyurethane material (MP 1880 Stevens Elastomerics). Polyurethane was selected because the ratio between its Young's modulus of elasticity and that of the brain stimulant (9,000–20,000) was similar to the ratio between the baboon falx and its brain (7,000–48,000). The dimensions of the falx were obtained from literature: thicknesses was 0.381 mm for the baboon and 0.508 mm the human (Davis and Huffman, 1968; Nieuwenhuys et al., 1978). The polyurethane falx was inserted and glued in the midsagittal plane such that the material was rigidly attached to the skull to simulate its firm connection to the tentorium and occiput.

Baboon (Papio nubis) skull-brain models with and without the falx, and adult human skull-brain model with the falx were subjected to biphasic acceleration-deceleration impulses with varying amplitudes and time durations using the HYGE® actuator and kinematic linkage system; the same apparatus was used in the authors' previous animal experiments (Gennarelli et al., 1982; Gennarelli et al., 1987). Figure 13.1 illustrates the mean peak acceleration/deceleration

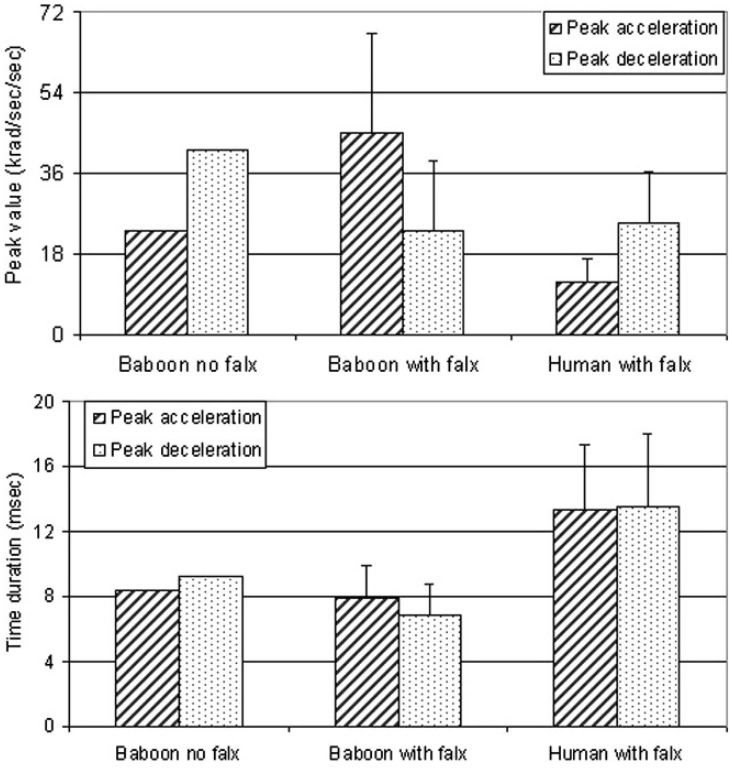


Fig. 13.1 Acceleration/deceleration pulse characteristics for the baboon and human skull-brain models. The *top* figure shows peak amplitudes. The *bottom* figure shows time durations. One test was conducted without the falx for the baboon and five tests each were conducted for the two models with the falx. Error bars represent standard deviations

pulse along with average time durations for each pulse. One test was conducted without the falx for the baboon, and five tests each were conducted for the baboon and human models with the falx. All physical models were accelerated through an arc of 65°. Simulated brain deformations in the three models were obtained by painting a grid pattern at spacing ranging from 1.5 to 3.0 mm and recording the motions with a film camera at 4,000–6,000 frames per second (Margulies et al., 1985, Margulies et al., 1990). Lagrangian angular displacements were determined by digitizing frames at 0.6 ms intervals (resolution 0.4 mm), and shear strains were calculated. Data were reported from eight tests.

Results for baboon models subjected to angular accelerations at 26.3 and 23.2 krad/s/s (angular decelerations: 46.9 and 41.4 krad/s/s) indicated decreases in intracranial deformations with the presence of the falx. Regions representing diffuse axonal injury locations in the human and baboon, i.e., tears in the corpus callosum, axonal damage and petechial hemorrhages in the white matter responded with decreased strains with the presence of the falx compared to the

Table 13.1 Summary of regional data from physical model experiments (Margulies et al., 1990)

Run	Model Type	Falx	Peak acceleration krad/s/s	Time ms	Peak deceleration krad/s/s	Time ms	Total time ms	Peak strain
1	Baboon	No	23.20	8.40	41.30	9.20	17.60	0.380
2	Baboon	Yes	17.50	10.70	31.00	9.50	20.20	0.073
3	Baboon	Yes	26.30	9.20	46.90	8.20	17.40	0.054
4	Baboon	Yes	52.20	7.10	10.40	5.70	12.80	0.088
5	Baboon	Yes	59.10	6.50	12.10	5.40	11.90	0.131
6	Baboon	Yes	69.60	6.30	15.80	5.50	11.80	N/A
	Mean		44.94	7.96	23.24	6.86	14.82	0.087
	SD		22.15	1.92	15.53	1.88	3.79	0.033
7	Human	Yes	3.70	20.20	7.60	21.40	41.60	0.052
8	Human	Yes	9.70	12.60	21.20	12.80	25.40	0.109
9	Human	Yes	12.00	12.20	25.10	11.60	23.80	0.096
10	Human	Yes	15.40	11.30	31.10	10.90	22.20	0.128
11	Human	Yes	17.70	10.30	38.90	10.90	21.20	N/A
	Mean		11.70	13.32	24.78	13.52	26.84	0.096
	SD		5.43	3.95	11.70	4.47	8.40	0.032

strains with the absence of the falx. Table 13.1 shows regional strain data along with input acceleration-deceleration pulse details for all the three models in all tests. Although not shown, in contrast, strains in the region representing the center of mass of the model were small and were insensitive to the presence or absence of the falx. Peak strains increased with increasing levels of angular loading, and occurred in the deceleration or unloading phase of the biphasic pulse. Similar results were found for the human skull-brain physical model. These physical modeling studies indicated the protective effect of the falx on certain brain components via strain metrics.

13.3 The Role of Falx Using Finite Element Models Under Angular Acceleration

In order to study the effects of inertial acceleration on regional brain strains, recently Li et al. developed a two-dimensional computational model of the human skull-brain system (Li et al., 2007). The finite element model was based on physical experimental data (Bradshaw et al., 2001). The physical model geometry and anatomy replicated adult human head, and the dimensions were obtained from magnetic resonance images of ten subjects. The computational model consisted of circular rigid skull, falx, cerebrospinal fluid, and cerebrum (Fig. 13.2). The skull was 192 mm in internal diameter, the falx was 8 mm wide and 60 mm deep, and a three-quarter circle represented the 2.5 mm thick cerebrospinal fluid layer. The aluminum skull and falx were assumed linear elastic: Young's modulus 70.3 GPa, Poisson's ratio 0.345, and density

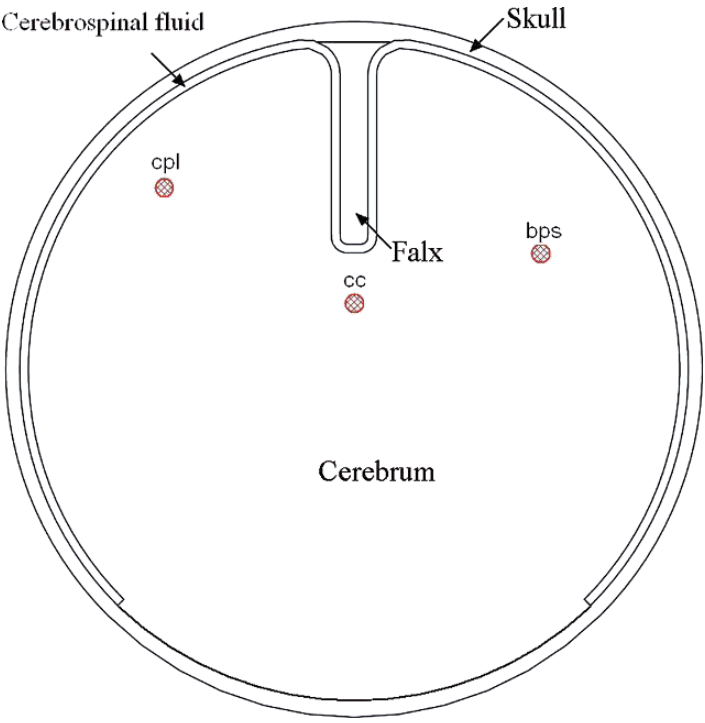


Fig. 13.2 Finite element model identifying the corpus callosum (cc), the base of the postcentral sulcus (pcs), and the cerebral cortex of parietal lobe (cpl) where motions were extracted

2,700 kg/m^3. The liquid paraffin simulated the cerebrospinal fluid, and the silicone gel simulated the brain. Both components were considered nearly incompressible. For the liquid paraffin, a hydrodynamic material model, governed by the Mie-Grüneisen equation of state in linear Hugoniot form, was used. A linear viscoelastic material law was adopted for the silicone gel. Material properties were obtained from literature (Bradshaw et al., 2001, Meyers and Chawla, 1984). The 4,650 element model shown in Fig. 13.3 was meshed by a preprocessor (MSC Patran, Version 2005-r2, MSC Inc., Santa Ana, CA, USA). The outer nodes of the skull were rotated about the center of geometry. The maximum angular acceleration was 7800 rad/s^2 with 4.5 ms duration (Fig. 13.4). A nonlinear explicit finite element software (ABAQUS version 6.5, HKS Inc., Providence, RI, USA) was used for extracting the output of the stress analysis. The model was validated against experimental temporal motion and strain data. Motions and strains were obtained at the corpus callosum, base of the postcentral sulcus, and cerebral cortex of the parietal lobe (Fig. 13.2). Data were extracted for two cases: rigid and flexible falx. The flexible falx was represented with Young's modulus of 10 MPa while the material properties of all other components remained unchanged.

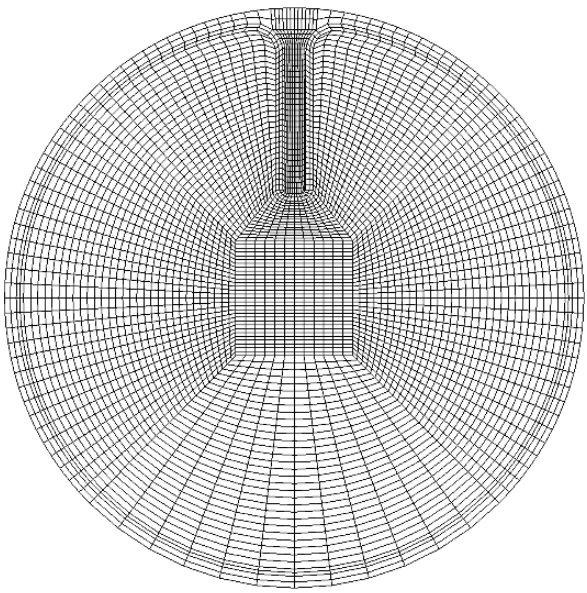


Fig. 13.3 Finite element mesh used to study the effect of the rigidity of the falx on brain strains

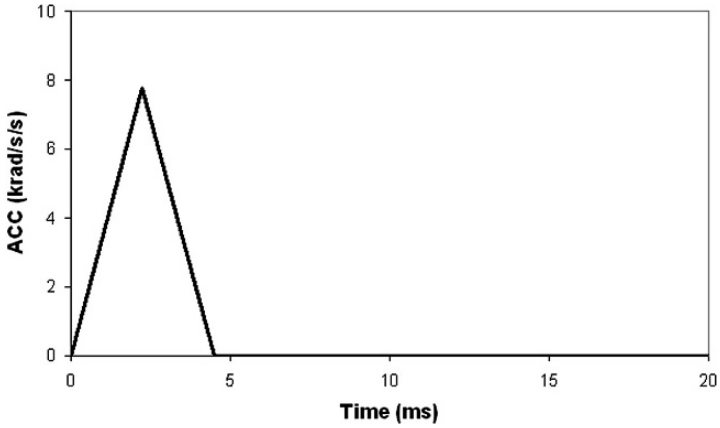


Fig. 13.4 Acceleration pulse used in the simulations

Results indicated that displacements in the corpus callosum to be the greatest under both cases (Fig. 13.5a). This was followed by displacements at the base of the postcentral sulcus, and the cerebral cortex of parietal lobe (Fig. 13.5b). A similar phenomenon was apparent for the principal strains in all regions (Fig. 13.6). The periphery of the brain surrounded by the cerebrospinal fluid sustained very low displacements and strains. In addition, although not discussed in the present context, greater level of strains occurred in the caudal region, presumably due to boundary condition effects. An examination of the strain distributions in the brain at the time of attainment of peak strains in the

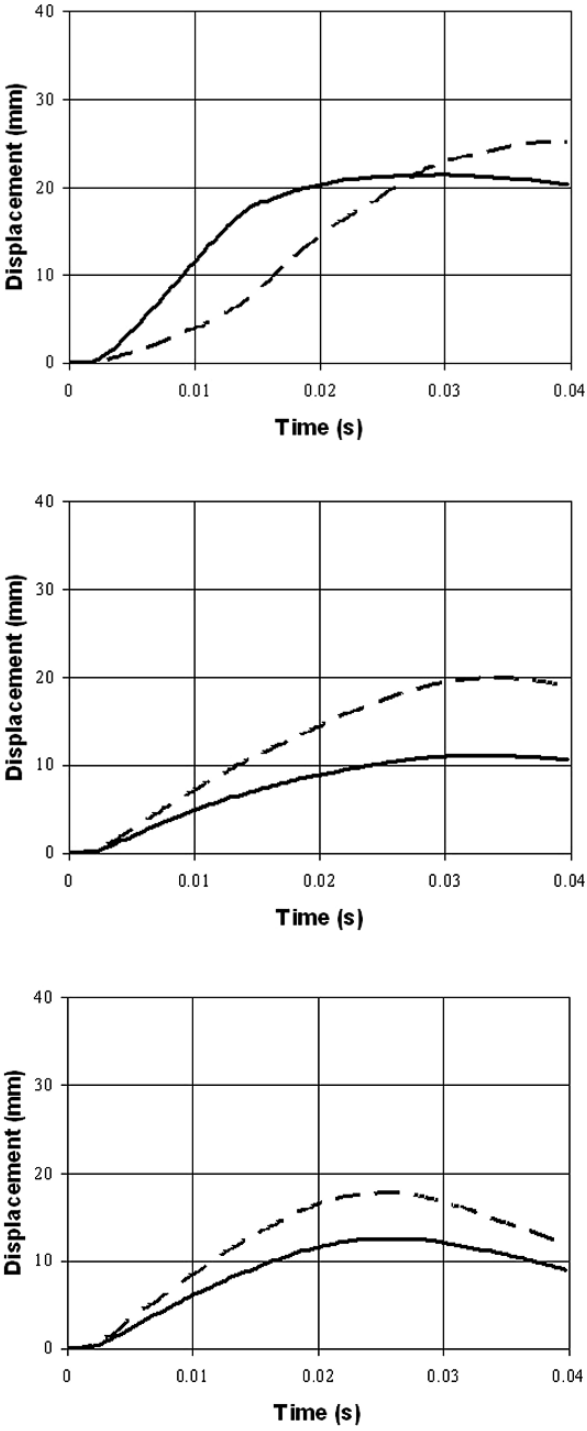
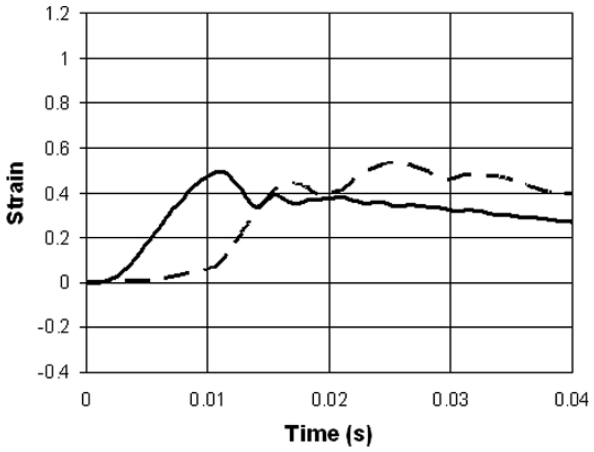
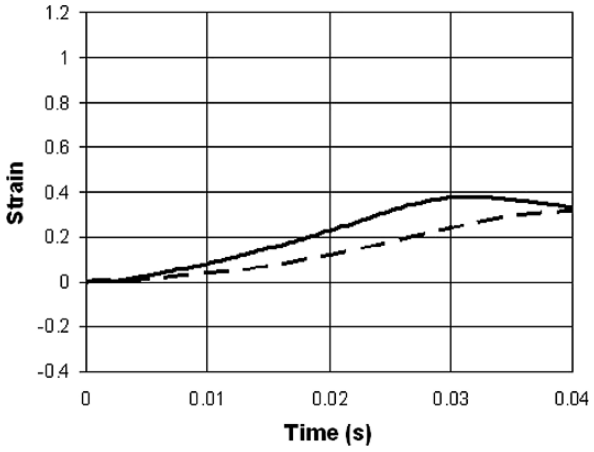


Fig. 13.5 (**A**) Temporal distributions of displacements at the corpus callosum. The *dashed curve* represents the response with flexible and *solid curve* represent the response with rigid falx. (**B**) Temporal distributions of displacements at the base of the postcentral sulcus (*top*) and at the cerebral cortex of parietal lobe (*bottom*). The *dashed curve* represents the response with flexible and *solid curve* represent the response with rigid falx





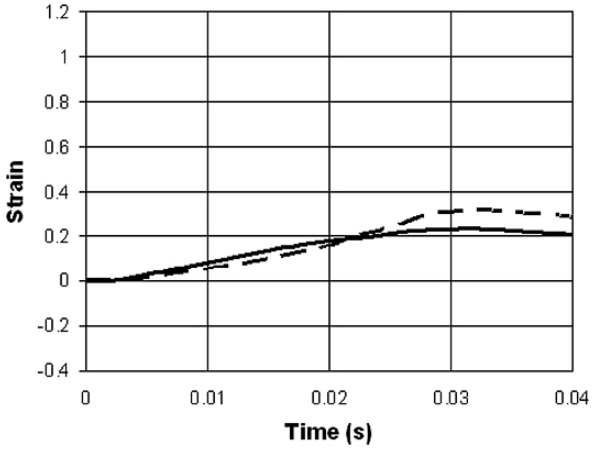


Fig. 13.6 (**A**) Temporal distributions of principal strains at the corpus callosum. The *dashed curve* represents the response with flexible and *solid curve* represent the response with rigid falx. (**B**) Temporal distributions of principal strains at the base of the postcentral sulcus (*top*) and at the cerebral cortex of parietal lobe (*bottom*). The *dashed curve* represents the response with flexible and *solid curve* represent the response with rigid falx

corpus callosum indicated a more focal pattern for the case of rigid falx than the flexible falx (Fig. 13.7). This pattern was not fully repeated when the strain distribution was examined at the time of attainment of peak strains at the other two components (Fig. 13.8 shows the distribution at the base of the post central sulcus), demonstrating the regional effect of the falx.

13.4 The Role of Falx Using Finite Element Models Under Contact-Induced Impact Loads

In order to study the effects of direct impact-induced loading on regional brain stresses, a two-dimensional computational model of the human skull-brain system was used (Nishimoto and Murakami, 1998). The two-dimensional

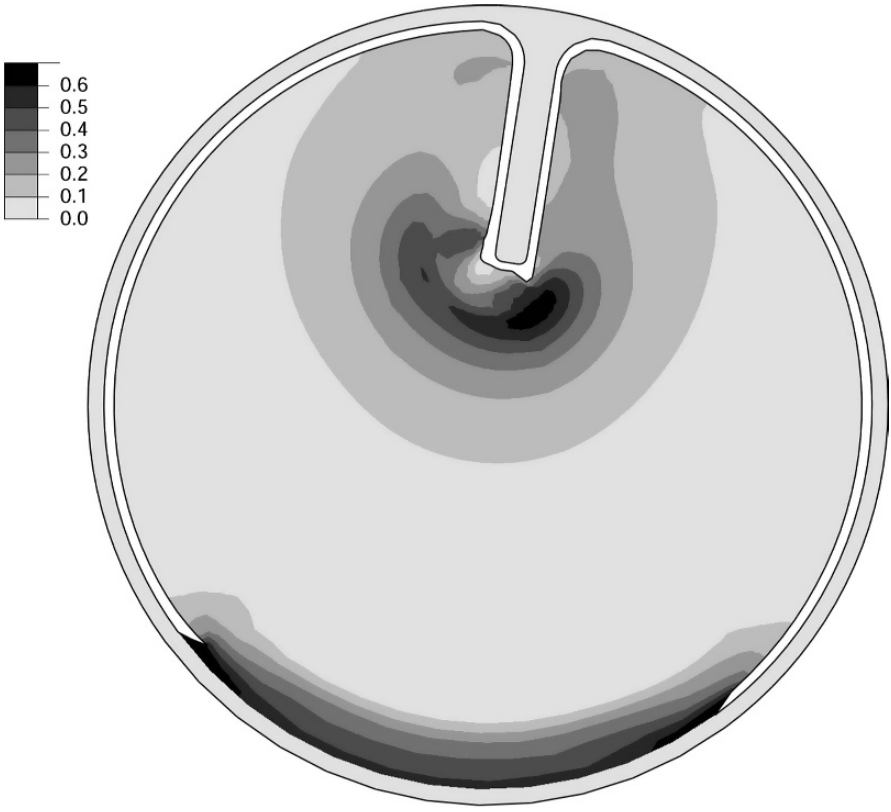


Fig. 13.7 *Top* figure shows the strain pattern in the brain at 11.5 ms for the case of rigid falx when the strain in the corpus callosum reaches its maximum magnitude. The *bottom* figure corresponds to the case without falx at 25.5 ms when the strain reaches its peak magnitude at the corpus callosum

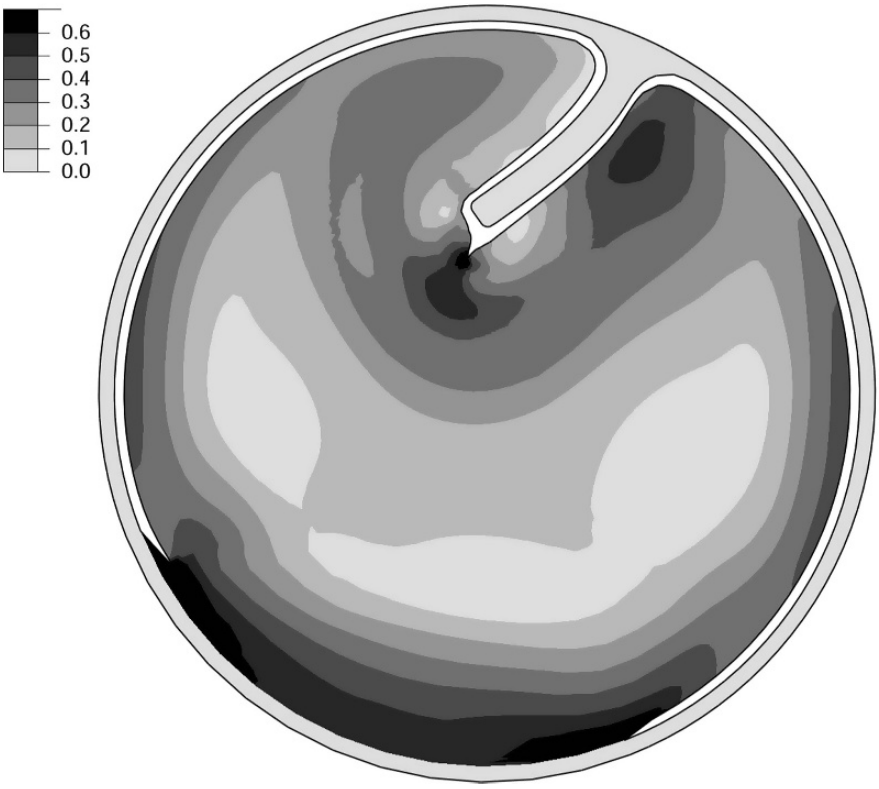


Fig. 13.7 (continued)

model representing a coronal slice simulated the falx, tentorium, dura matter, cerebrospinal fluid, ventricles, interpeduncular cistern, gyrus, sulcus, and skull. The cortical bone was simulated as an elastic-plastic material, and its mechanical properties were based on authors' experiments. The brain, cerebrospinal fluid, ventricles, and cistern were simulated as linear viscoelastic solids. While the brain was simulated by bulk modulus of 2.19 GPa, short- and long-term shear modulus of 0.000528 and 0.000168 GPa, and decay constant of $35\,s^{-1}$, the cerebrospinal fluid, ventricles, and cistern were represented by bulk modulus of 0.0219 GPa, short- and long-term shear modulus of 0.000528 and 0.0005 GPa, and decay constant of $5\,s^{-1}$. Density of 1040 kg/m^3 was used to simulate all these materials. These data were based on mechanical characteristics from literature (Galford and McElhaney, 1970; McElhaney et al., 1970). The falx cerebri, tentorium cerebelli and dura matter followed the material properties of the cranium. The sagitally constrained human model consisted of 11,472 nodes and 7,558 solid elements. All degrees-of-freedom were unconstrained at the base of brain stem. Using the PAM-CRASH explicit finite element software

and Lagrangian formulation, the model was exercised using side impact loading toward the third ventricle.

The model was validated using human cadaver intracranial pressure data and another finite element model results (Nahum et al., 1977; Nahum et al., 1980; Ruan et al., 1993). The two-dimensional model was exercised at 2.0 m/s velocity with an impactor mass of 1.56 kg (kinetic energy 3.12 Nm) and 6 ms duration, produced a peak force of 0.19 kN, coup pressure of 99.3 kPa and contrecoup pressure of −77.0 kPa. The three-dimensional human cadaver experiment used in the validation process was conducted at a velocity of 7.03 m/s, with an impactor mass of 4.17 kg (kinetic energy 103 Nm); the test produced coup and contrecoup pressures of 49.6 and −95.7 kPa (Nahum et al., 1980). Another finite model, exercised at a velocity of 6.33 m/s, with an impactor mass of 5.23 kg (kinetic energy 105 Nm) produced coup and contrecoup pressures of 265.0 and −85.0 kPa (Ruan et al., 1993). Despite these

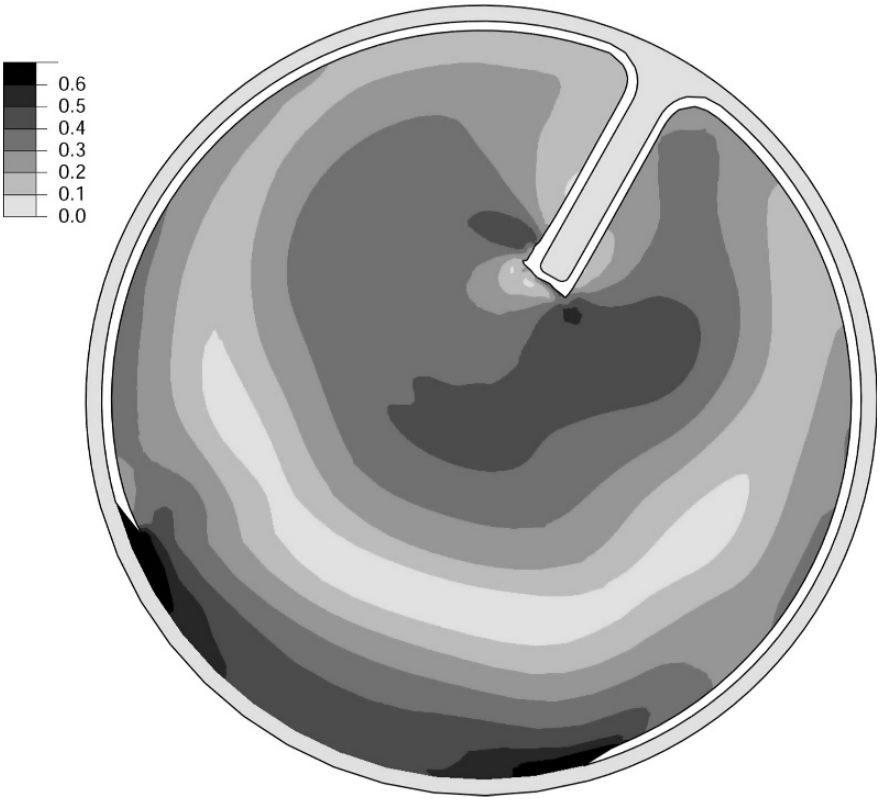


Fig. 13.8 *Top* figure shows the strain pattern in the brain at 31.5 ms for the case of rigid falx when the strain in the base of the postcentral sulcus reaches its maximum magnitude. The *bottom* figure corresponds to the case with flexible falx at 32.5 ms when the strain reaches its peak magnitude at this region

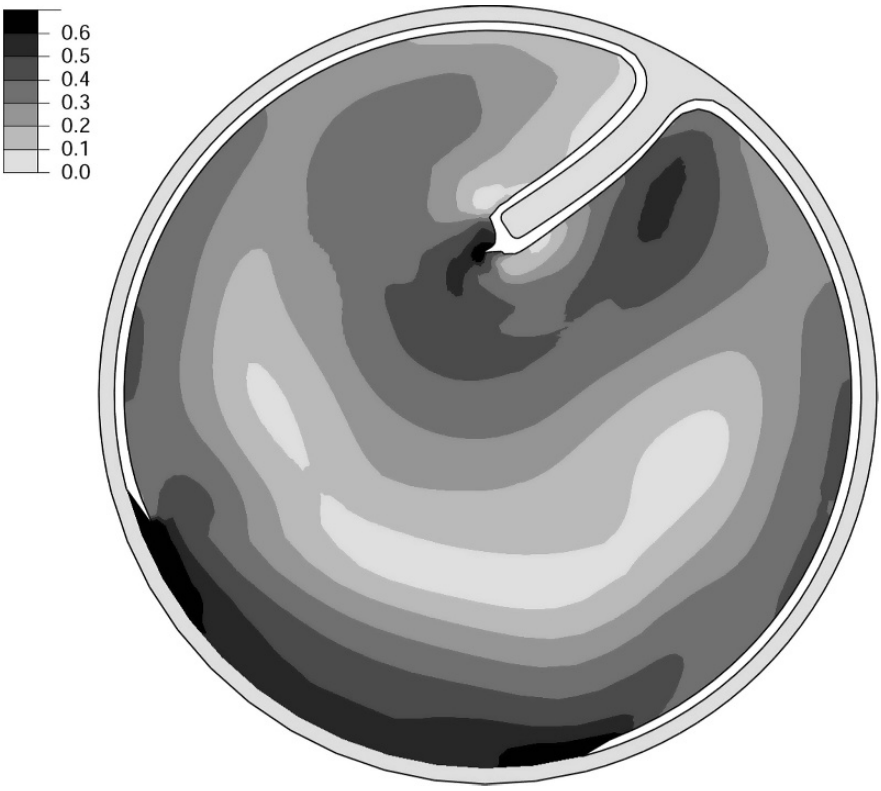


Fig. 13.8 (continued)

differences in the output, and acknowledging that the dimensionalities of the models were varied, the authors' judged the model to be validated, and presented analysis of stresses from the human model and a modified model without the simulation of the falx cerebri.

The model without the simulation of the falx cerebri, consisted of 10,878 nodes and 7,174 elements. For both models, shear stress distributions were determined. Maximum shear stresses at the location corresponding to the corpus callosum were approximately 50% lower in the model without the falx than in the intact human model with the intact falx (Fig. 13.9). In the rostral brainstem area, however, both models produced similar magnitudes of shear stress (Fig. 13.9). Without the falx, peak stresses were delayed in the rostral brainstem area while such delay was not apparent for the location corresponding to the corpus callosum (Fig. 13.10). Temporal lobes on the ipsilateral and contralateral regions produced much lower peaks in both models (not shown), suggesting the relative insensitivity to the presence of falx. All stress patterns were oscillatory due to the type of the load vector, i.e., short-duration impact to

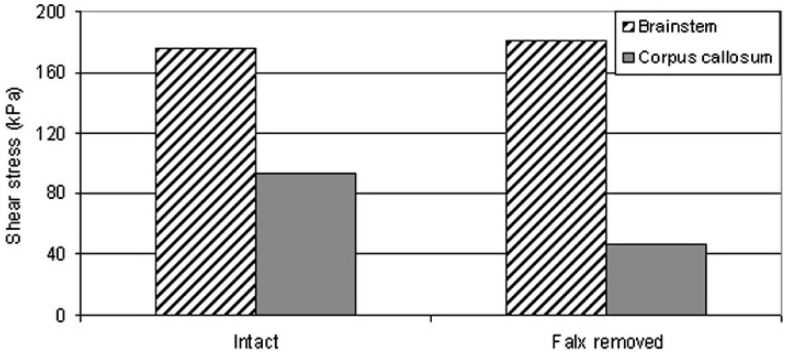


Fig. 13.9 Peak magnitudes of shear stress with (intact) and without the falx in the brainstem and corpus callosum under contact-induced impact loading

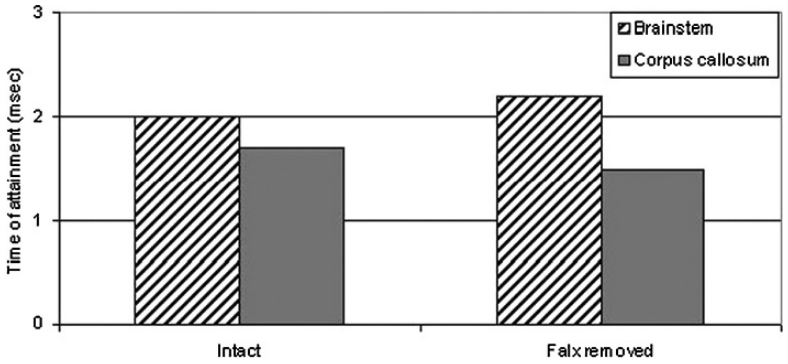


Fig. 13.10 Time of attainment of peak magnitudes of shear stress with (intact) and without the falx in the brainstem and corpus callosum under contact-induced impact loading

the skull. While peak stresses in the skull (2.3 MPa) were an order of magnitude greater than the brain, peak values did not reach fracture limits (yield stress 41.8 MPa), suggesting no bony fracture. Although the study reported tensile distributions data for the intact model, i.e., peak values of 0.098 and 0.050 at the rostral brainstem area and location corresponding to the corpus callosum, data were not reported for the model without the falx. Despite this lack of data, these analyses indicate that the falx cerebri affects regional stress and strain patterns in terms of magnitude and duration under contact-induced impact loads.

13.5 Conclusion

As described in the Introduction, the purpose of this chapter was to present information on the potential role of falx on internal brain mechanics during dynamic loads. Physical and computational modeling studies were used to

present literature data. This is because while animal modeling studies provide the critical information on brain pathology and function, region-specific intrinsic mechanical metrics cannot be directly determined from experiments. Likewise, clinical studies including autopsy and histological procedures are incapable of delineating the intrinsic mechanics, albeit these studies are invaluable to understand the consequences of external mechanical dynamic loads. In fact, for over fifty years, a plethora of investigations have identified susceptible structures (Adams et al., 1980, 1982, 1991; Gennarelli, 1985; Gennarelli and Thibault, 1982; Gennarelli et al., 1982, 1987, 1989; McLean, 1995; Ommaya and Gennarelli, 1974; Peerless and Rewcastle, 1967; Strich, 1956; Strich, 1961; Thibault and Gennarelli, 1985; Unterharnscheidt and Higgins, 1969; Unterharnscheidt and Higgins, 1969). Despite the shortcomings, experimental animal and clinical investigations play an important role for advancing physical and mathematical models. Specifically, the latter types of models are validated by examining simulated internal structures of the brain, example, corpus callosum, for peak values of stresses and strains. As described in earlier paragraphs, physical models of the baboon and adult human have used animal and patient data.

Localized regions within the physical models were selected in the reviewed literature for stress-strain analyses using animal and human pathologic data that produced diffuse brain injuries. These included classical sites of diffuse axonal injuries, i.e., tears in the corpus callosum, areas of axonal damage, and petechial hemorrhage in the white matter; rostral brain stem, as well as sites less frequent for such trauma (Adams et al., 1982; Gennarelli et al., 1982; Peerless and Rewcastle, 1967; Strich, 1956; Strich, 1961). A close correlation between peak strains in the physical model at these clinically relevant regions and sites of pathology in biological tissues assisted in the validation process. In contrast, computational models relied on physical modeling deformation results, coup and contrecoup pressures results from human cadaver experiments, and other finite element results (Bradshaw et al., 2001; Li et al., 2007; Nahum et al., 1977, 1980; Nishimoto and Murakami, 1998; Ruan et al., 1993). In addition, computational models used to delineate the role of falx also relied on clinical information from previously cited literature. While these approaches have served validation processes, full validation is yet to be achieved. Intracranial pressures from human cadavers tend to offer a more "gross" validation as many parameters are not available from studies, performed approximately three decades ago. Although more recent human cadaver studies have reported local deformations using advanced transducer technologies, these data are yet to be exploited by modelers to delineate the role of falx (Zhang et al., 2001) . This may be a topic for future research.

Regional brain strains were extracted from the stress analysis output under inertial biphasic acceleration loading as these were directly attributed to be the cause of diffuse brain injuries in experimental animal studies (Li et al., 2007). In contrast, shear stresses were extracted from models using contact-induced impact loads because the evaluation of direct impact by this variable is equivalent to the evaluation of strains due to angular acceleration (Nishimoto and

Murakami, 1998). Consequently, these biomechanical metrics were used in delineating the role of falx on brain dynamics. It should however be noted that, while not specific to falx, modeling results, applying insults from 33 full-scale motor vehicle tests conducted by the US Department of Transportation, have shown that internal brain strains are more sensitive to angular than linear accelerations (Zhang et al., 2006). In this study, a simplified version of the human finite element head model, (Simulated Injury Monitor SIMon), was used. The SIMon has been validated with, and injury metrics, cited above, have been derived using experiments (Takhounts et al., 2003). Strains at multiple brain elements, cumulative strain damage, dilatation damage, and relative motion damage data were evaluated (Zhang et al., 2006). Rotational accelerations contributed to more than 80% of the brain strain. These findings did not depend on the crash mode, frontal or side impact, peak amplitude of translational (29–120 g), or angular acceleration (1.3–9.4 krad/s^2), or head injury criteria (68–778). Such analyses can also be pursued to evaluate the role of falx in motor vehicle environments.

In general, the presence of falx that can be considered as a medium differentiating the two hemispheres, tend to alter the internal stress-strain distributions due to inertial- or contact-induced dynamic loading. Under the latter type of loading, stresses in the brainstem (and bilateral temporal lobes) were relatively insensitive to the presence or absence of falx, and stresses were lower at the corpus callosum in the model without falx. In contrast, under the former type of loading, the discussed parametric study showed high strain concentrations in the corpus callosum area for the rigid falx, while high strains extended into the cerebral vertex in the model with the flexible falx. It is important to consider the falx as a flexible connecting medium within the brain to predict internal strain fields due to mechanical insults such as lateral impact acceleration. From a mechanics perspective, the flexible falx redistributes/extends the local strain field into regions beyond the corpus callosum, thus emphasizing its role in diffuse axonal injuries. These types of injuries are routinely identified as stemming from prolonged loss of consciousness together with radiological identifications of abnormalities representing diffuse areas within the brain. These computational modeling results should be considered as a first step in determining the role played by this important component of the brain structure. Parametric studies such as including the three-dimensional geometric features of the complex human skull-brain system and loading modes other than pure lateral impacts are needed to better understand the intrinsic brain biomechanics and injury due to dynamic loads.

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Chapter 14
Exercise and Nervous System

Kazuhiro Imai and Hiroyuki Nakajima

Abstract Voluntary physical training and exercise have favorable effects on the central nervous system and brain plasticity. The motor cortex and spinal cord possess the ability to alter structure and function in response to motor training. It is also reported that intensive training and exercise may enhance motor recovery or even restore motor function in people who have been long paralyzed due to spinal cord injury or stroke. We review the effects of exercise on the nervous system and discuss the mechanism for the exercise effects.

Keywords Exercise · physical activity · central nervous system · brain plasticity · sympathetic nervous system · corticospinal plasticity

14.1 Introduction

Voluntary physical training and exercise have profound effects on numerous biologic systems within the human body, including the cardiovascular, pulmonary, musculoskeletal systems as well as the central nervous system (CNS). In contrast to our extensive knowledge about the peripheral adaptations to exercise, information about the specific effects of exercise on the CNS is relatively limited (Meeusen and DeMeirlier, 1995). The CNS can be considered the integrative center for all behavior. It receives and interprets sensory information from both the external word and the internal environment. During exercise, sensory feedback results in alterations in the CNS that can have profound effects on subsequent motor activity and psychologic function. This article introduces some of the neurobiologic changes during exercise.

There is growing evidence that increased physical activity reduces the risks of incident obesity, cardiovascular diseases, type 2 diabetes, osteoporosis, cancer, and depression (Dishman et al., 2004; U.S. Department of Health and Human

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Services, 1996). There is also growing evidence that physical activity confers health protective benefits for several neurological diseases including Parkinson's disease (Smith and Zigmond, 2003), Alzheimer's dementia (Cotman and Berchtold, 2002), and ischemic stroke (Stummer et al., 1994), as well as injuries from falls attributable to neuromuscular declines associated with physical inactivity among the elderly (Robertson et al., 2002). In contrast, relatively little is known about mechanisms of physical activity and exercise on the function of CNS.

14.2 Effects and Mechanisms of Exercise on Brain

14.2.1 Cognitive Functions

Chronic physical activity improves brain health. Among humans, regular exercise has a beneficial impact on depression (Blumenthal et al., 1999; Dunn et al., 2001; Lawlor and Hopker, 2001), quality of sleep (Driver and Taylor, 2000), and cognitive function in older adults (Colcombe and Kramer, 2003; Hillman et al., 2002; Kramer et al., 1999; van Gelder et al., 2004; Weuve et al., 2004). Both long-term research from cross-sectional studies and short-term research from randomized clinical trials suggest positive influences of physical activity and fitness training on human brain structure and function. Some evidence further suggests that there can be beneficial effects of exercise on cognition across the life span.

14.2.2 Neurotrophic Effects

Chronic physical activity increases the expression of brain growth factors. In rats, chronic exercise can increase the expression of genes that encode several brain neurotrophins such as brain-derived neurotrophic factor (BDNF) (Neeper et al., 1995; Oliff et al., 1998) and nerve growth factor (Ang et al., 2003). Chronic physical activity may also have neurogenerative and neuroprotective influences on the brain by stimulating the growth and development of new cells (Rhodes et al., 2003) and protecting against ischemic neuronal damage in the hippocampal formation (Stummer et al., 1994) and neurotoxic damage in the neostriatum (Döbrössy and Dunnett, 2003).

Some putative mechanisms by which exercise affects cognition have been identified: learning capacity is mediated by the action of BDNF on synaptic plasticity, which has the potential to underline cognition (Gómez-Pinilla et al., 2002). Only 1 week of voluntary wheel running can increase the capacity for learning and memory in rats (Vaynman et al., 2004). It is now understood that the action of exercise on the BDNF system modulates the function of intracellular signaling systems such as calcium-calmodulin kinase 2 and

mitogen-activated protein kinase. Enhanced learning after chronic wheel running has also been accompanied by decreases in extracellular amyloid-β plaques and proteolytic fragments of amyloid precursor protein in a transgenic mouse model of Alzheimer's disease (Adlard et al., 2005). It has been found that exercise significantly increases the levels of the mitochondrial uncoupling protein 2, an energy-balancing factor concerned with the maintenance of calcium homeostasis, ATP production, and free radical management (Vaynman et al., 2006). Uncoupling protein 2 seems to modulate BDNF production by hippocampal cells, as well as molecular systems downstream to BDNF action that is important for learning and memory. These results suggest fundamental mechanisms by which exercise affects key elements of energy metabolism that modulate substrates of synaptic plasticity underlying learning and memory. Treadmill exercise training has been accompanied by increased gene expression for BDNF and nerve growth factor concomitantly with reduced brain infarct volume after focal ischemia by middle cerebral artery occlusion in rats (Ding et al., 2004; Li et al., 2004), and it has mitigated caspase-dependent apoptosis in hippocampal neurons after transient global ischemia in the gerbil (Lee et al., 2003). Exercise can also improve the outcome of traumatic brain injury, but the timing and the intensity is important for effectiveness. Exercise provided 2 weeks after concussive brain injury attenuates the effects of the injury through a BDNF-mediated mechanism (Griesbach GS et al., 2004). Neuroprotection by prior locomotory training against chemical lesion of nigro-neostriatal dopamine neurons seems to result from an increase in the availability of the trophic factor, glial cell line-derived neurotrophic factor, which can stimulate certain cell signaling cascades, including the activation of extracellularly regulated kinases.

14.2.3 Mechanisms of Exercise on Central Nervous System

An understanding of the influence of the CNS on motor neurons, especially limitations to neuromuscular performance is emerging (Noakes et al., 2005). Corollaries of motor command are available at multiple levels in the CNS and are likely to sustain multiple functions (e.g., motor, sensory, cardiovascular, respiratory, hormonal) during physical activity. However, their influences on CNS functions (e.g., executive cognitive function, learning, emotional responding, motivated behavior) other than those dedicated to controlling locomotion and supporting its resultant increase in metabolism are not known and have received little study. Several metabolic and neurochemical pathways among skeletal muscle, the spinal cord, and the brain suggest ways by which physical activity and exercise might influence the CNS. Lactate taken up from skeletal muscle seems to act as an intercellular energy shuttle within the brain during high-intensity exercise and changes in oxidative enzymes in skeletal muscle after exercise could have indirect effects on brain metabolism (Dalsgaard

et al., 2004). Both short-term high-intensity and prolonged low-intensity exercise increase pyruvate dehydrogenase kinase-4 transcription (PDK4) in active skeletal muscle, thus inhibiting the entry of carbohydrate-derived fuel into the mitochondria for oxidation and limiting the use of glucose as a fuel for skeletal muscle, subsequently protecting the increased metabolic needs of the brain during and after exercise (Pilegaard et al., 2000). Transient increases in local cerebral glucose use in the motor cortex, cerebellum, striatum, thalamus, hippocampus, somatosensory, auditory, and visual cortices have been reported in response to acute strenuous treadmill running in rats (Vissing et al., 1996). The discharge rate of a select pool of hippocampal cells has been observed to increase as running velocity increases (Czurko et al., 1999). Oxidative capacity after chronic voluntary activity wheel running is increased only in the striatum and limb representations in the motor cortex and not in the hippocampus of rats (McCloskey et al., 2001). In humans, cerebral blood flow is transiently increased in the thalamus, anterior cingulate, insula, and sensorimotor cortex during static handgrip (Williamson et al., 2003) and in the thalamus and insula during dynamic cycling exercise (Williamson et al., 1997).

The influence of central motor command on autonomic cardiovascular responses during exercise has been shown by increases in perceived exertion, blood pressure, and heart rate, concomitantly with increased cerebral blood flow to insular and thalamic regions, after hypnotic suggestion of increased work during constant-load cycling exercise (Williamson et al., 2001). Epidural anesthesia sufficient to cause cutaneous sensory loss and paralysis of the legs during electrically induced dynamic exercise suggests that neural and humoral mechanisms exert redundant control of circulating catecholamines, growth hormone, and adrenocorticotropic hormone. In contrast, neural input from motor centers and feedback from active muscles both seem important for glucose production and lipolysis during exercise in humans (Kjaer et al., 1996). Also, during complete paralysis by heart rate, blood pressure, and perceived effort still increase during attempted contraction of skeletal muscles, indicating that central motor command can operate independently of somatosensory inflow to the CNS (Gandevia et al., 1993).

Neurobiological aspects of the regulation of physical activity by the CNS are poorly understood. Homo Sapiens has evolved a large cerebral cortex with expanded motor and executive regions, as well as an elaborate distribution of direct and indirect corticospinal connections with motor neurons that supports prolonged physical activity such as sustained running (Bramble and Lieberman, 2004). One consequence of this evolution is the ability, through central drivers from the motor cortex, locomotory circuits, or motor neurons, to override strong somatic, visceral, and homeostatic circuits, which would stop ongoing exercise. Putative brain mechanisms of impaired voluntary activation of motor neurons during prolonged strenuous exercise include an effective decrease in supraspinal motor drive to motor neurons, which may be associated with increased brain 5-HT activity, elevated ammonia levels, brain glycogen

depletion, decreased striatal dopamine, and inhibitory feedback from the exercising muscles (Nybo and Secher, 2004).

It is known that spinal motor learning that is specific to a practiced task can occur within the neuronal networks in the lumbosacral spinal cord in mice, rats, cats, and humans that have a transected spinal cord (Edgerton et al., 2004). This learning involves the two major inhibitory neurotransmitter systems within the spinal cord: glycine and gamma amino butyric acid. Spinal cord transaction results in augmentation of these systems above normal levels, but training to step reduces these levels back toward normal levels. Administration of 5-HT agonists enhances stepping in mice that have complete spinal transections and facilitates learning to step when combined with training. The functional properties of the lumbosacral spinal cord and the general mechanisms by which locomotion is controlled in humans (Crameri et al., 2004) seem to be qualitatively similar to those described for several laboratory animals (Edgerton and Roy, 2002; Edgerton et al., 2002; Tillakaratne et al., 2002; Timoszyk et al., 2002).

The effects of neural activation of skeletal muscle are also becoming clearer. As few as 4 minutes of daily total stimulation, under isometric conditions, of otherwise electrically silent skeletal muscles are sufficient to maintain the mass of a fast extensor muscle. The importance of the motor activity associated with the support of body weight seems to be critical in maintaining basal levels of neurotrophins and neuroplasticity in the spinal cord (Gómez-Pinilla et al., 2004) and the body's normal endocrine responses to exercise.

Reduced dopamine release or loss of dopamine receptors in the brain seems to be related to the age-related decline in physical activity observed among many species (Ingram, 2000). The ventral-tegmental-nucleus accumbens dopamine system is a critical component of the forebrain circuitry that regulated activational aspects of motivation. Accumbens dopamine depletions decrease spontaneous, stimulant-induced, and food-induced motor activity, depending on the work requirements of the task. Antagonists of dopamine and depletions of accumbens dopamine cause rats to reallocate their instrumental behavior away from food-reinforced tasks that have high work requirements and toward the selection of less effortful types of food-seeking (Salamone et al., 2003), implicating dopamine brain circuitry in energy-related decision-making.

Electrical self-stimulation of the ventral tegmental area has been used to motivate treadmill running (Burgess et al., 1991) and weight lifting (Garner et al., 1991) in rats. Treadmill running acutely increases dopamine release (Meeusen et al., 2001) and turnover (Hattori et al., 1994) and chronically upregulates D2 receptors (MacRae et al., 1987) in the striatum of rats, but the effects of exercise on striatal dopamine activity have not yet been shown in humans (Wang et al., 2000).

Physical activity is bodily movement produced by skeletal muscles that results in varying amounts and rates of energy expenditure that are positively related to physical fitness depending on the stimulus features of physical activity such as the type, intensity, regularity, and timing of the activity. Physical

activity can occur in short bursts of low to high intensity or long sustained periods of lower intensity, depending on the type of activity and the fitness of the organism. Exercise is a specific form of physical activity that is structured and repetitive, with the goal of improving or maintaining physical fitness, function or health.

Outflow from the CNS to organ systems that support locomotion for a particular task must change over time as the musculoskeletal system changes in response to changes in the amounts or types of physical activity. Hence, it necessarily follows that some of the CNS changes in response to physical activity must directly reflect the altered central drive to execute the same external task. This fact adds to the difficulty of interpreting all CNS changes that accompany chronic physical activity sufficient to elicit changes in exercise capacity.

As for the psychologic effects, regular physical activity can change a person's perception of his or her physical self and identity in a positive way and it can also be used as a means to reduce stress and anxiety (Brosse et al., 2002; Fox, 1999). Experimental studies support a positive effect on mood for moderate-intensity exercise (Brosse et al., 2002) and numerous cross-sectional and longitudinal studies have demonstrated the beneficial effects of regular exercise on the clinical course of several depressive disorders, including major depressive disorder and minor depression (Meyer and Broocks, 2000).

Several hypotheses have been developed to try to explain the mechanisms by which exercise can exert beneficial effects on depressive disorders. One theory that has received considerable attention is the β-endorphin hypothesis. β-endorphin is an endogenous opioid released by the anterior pituitary. The mood elevation described by many athletes in response to prolonged exercise, such as the "runner's high" reported by athletes participating in long-distance running, had previously been attributed the release of endogenous opiates such as β-endorphin (Janal et al., 1984). Initial support for the hypothesis that β-endorphin contributes to the antidepressant effects of exercise was derived from studies demonstrating an association between post-exercise mood elevations and increases in circulating β-endorphin levels (Paluska and Schwenk, 2000). Additionally, improvements in mood that occur with an acute bout of endurance exercise can be reduced when the opioid antagonist naloxone in administered (Janal et al., 1984). Despite evidence that exercise-induced increases in β-endorphin levels are associated with short-term mood enhancement, it is not conclusive that these changes result in more sustained effects (Paluska and Schwenk, 2000). Several other theories have been suggested to explain the beneficial effects of exercise on mood. Both biologic and psychologic mechanisms have been proposed but none of them has been confirmed through rigorous scientific study. Given the complexity of the interaction between exercise and psychological function, an integrative biopsychosocial model that incorporates several mechanisms will likely provide the best explanation.

The potentially favorable neurobiologic effects of regular exercise have generated increased interest in the possible role of exercise to help preserve

cognitive function in older adults. Several prospective studies have demonstrated that physical activity is associated with a reduced risk of cognitive decline in older adults (Colcombe and Kramer, 2003; Hillman et al., 2002; Janal et al., 1984; van Gelder et al., 2004; Weuve et al., 2004). The benefits of exercise on the preservation of cognitive function extend beyond the ability of regular physical activity to reduce the risk of certain medical conditions that are associated with poor cognitive function in older adults, such as cardiovascular disease, cerebrovascular disease, hypertension, and diabetes mellitus (Barnes et al., 2003). Animal research has demonstrated that exercise can help preserve neuronal tissue, stimulate neurogenesis, and promote brain vascularization. These findings lend support to the concept that exercise has direct effects on the brain that may help to maintain brain function and promote brain plasticity.

It is obvious that the action of exercise on the CNS is mediated by multiple mechanisms involving highly integrated responses of all physiological systems. The study of mechanisms involved in CNS changes that are associated with exercise is essential for the timely development of more efficacious and rapid treatments for several neural and metabolic disorders. Understanding these mechanisms is perhaps even more important for identifying appropriate guidelines for physical activity and exercise that can positively affect public health (Anish, 2005; Dishman et al., 2006).

14.3 Effects of Exercise on Human Brain Structure

As compared with the study of the relationship between exercise and cognition, relatively few studies have been conducted to examine exercise effects on human brain structure and function. Changes in a neural network have been investigated by functional magnetic resonance imaging (fMRI) activation obtained in a high field magnet, over the course of a six month aerobic exercise program (Colcombe et al., 2004). Older adults performed the flanker task, which entails focusing on a subset of information presented on a visual display and ignoring task-irrelevant distractors, before and after the exercise training interventions. Individuals in the aerobic training group showed a reduced behavioral distraction effect and change in pattern of fMRI activation (i.e. increased right middle frontal gyrus and superior parietal activation) similar to that displayed by younger controls. Participants in the toning and stretching control group did not show such behavioral and fMRI changes. More recently, a semi-automated segmentation technique on high-resolution MRI was employed on older adults who were randomly assigned to either 6 months of an exercise training intervention or a toning and stretching control group (Colcombe et al., 2006). The older adults who walked three days a week for up to 1 hour/day for 6 months displayed increases in gray matter volume in the frontal and temporal cortex, as well as increases in the volume of anterior white matter. There were no volumetric increases for the nonaerobic control group or in a group of

college-student control participants. Although such structural changes as a function of an exercise intervention parallels cognitive improvements observed with 6 months of exercise, it is unknown whether increases in cortical volume are directly related to enhanced cognitive performance. In addition, an increase in MRI measures of cerebral blood volume in the dentate gyrus of the hippocampus was reported for middle-aged groups who participated in a 3 three month aerobic exercise program (Pereira et al., 2007). These cerebral blood volume changes were related to both improvements in cardiorespiratory fitness and performance on a test of verbal learning and memory. Increases in cerebral blood volume in a parallel study of exercising mice were found to be related to enhanced neurogenesis, suggesting that cerebral blood volume might serve as a biomarker for neurogenesis.

14.4 Effects of Exercise on Sympathetic Nervous System

Cardiovascular diseases, such as hypertension and heart failure are often associated with overactivity of the sympathetic nervous system (SNS) (Julius and Nesbitt, 1996; Schlaich et al., 2004). It has been suggested that increases in physical activity produce beneficial effects on the cardiovascular system in normal and diseased individuals via alterations in neural control of the circulation (Billman GE, 2002; Cornelissen and Fagard, 2005; Zucker et al., 2004). These effects include reductions in blood pressure and sympathetic outflow in humans as well as in animal models of exercise training (Tipton, 1991). Because morbidity and mortality in cardiovascular disease are often associated with elevation in SNS activity (Benedict et al., 1996), the beneficial effects of physical activity are likely related, in part to reductions in sympathetic activity. Understanding the mechanisms by which exercise training alters control of the SNS in health and disease is important for developing new strategies in the prevention and treatment of cardiovascular disease. In addition, a further understanding of these mechanisms may help promote public policy changes that aid in the reversal of the current trends in physical inactivity.

One of the difficulties in establishing a conclusive effect of physical activity on SNS activity in humans is likely due to the variety of studies that have attempted to address this seemingly straightforward question. It is clear that individual studies report a variety of effects of exercise training on measures of SNS activity (Cornelissen and Fagard, 2005). Exercise training may also influence increases in SNS activity during exercise and in response to other stressors that cause sympathoexcitation, including baroreceptor unloading (Ray and Hume, 1998). Furthermore, the type of measurement used to assess SNS activity may influence conclusions on the effects of physical activity in SNS regulation. Most studies have used one or more of three basic techniques to assess resting SNS activity: plasma noradrenaline (NA) levels, regional NA spillover or direct microneurographic recordings from nerves innervating skeletal muscle

(i.e. muscle sympathetic nerve activity; MSNA). The significant effects of exercise training on SNS activity may also be influenced by additional factors. These include intersubject variability (Grassi et al., 1994), age and gender (Ng AV et al., 1994), previous and attained fitness levels (Fadel et al., 2001) and different degrees of adiposity in trained and sedentary subjects (Alvarez et al., 2005).

In contrast with somewhat equivocal results in humans, evidence from animal studies suggests that increases in physical activity produce consistent reductions in resting and reflex control of sympathetic outflow in normal animals (Krieger et al., 2001). Studies of exercise training in rats (Krieger et al., 1999) and rabbits (DiCarlo and Bishop, 1988; DiCarlo and Bishop, 1990) indicate a suppression of resting and baroreflex-mediated increases in renal sympathetic nerve activity. In addition to reduction in renal sympathetic nerve activity, previous studies have also reported blunted baroreflex activation of lumbar sympathetic nerve activity in spontaneous wheel-running rats (Chen and DiCarlo, 1996). There is also indirect evidence that treadmill training in rats reduces cardiac sympathetic nerve activity (Negrao et al., 1992). Therefore, data from well-controlled animal studies appear to indicate a more global effect of exercise training on SNS activity.

14.5 Physical Activity Dependent Plasticity in the Central Nervous System

Increasing scientific evidence is emerging that supports the existence of physical activity dependent plasticity in the CNS. Of particular interest is functional improvement such as reported for memory and cognition that are associated with changes in the number, structure and function of neurons (Anderson et al., 2000; Ang et al., 2006; Fordyce and Farrar, 1991,a,b; van Praag et al., 1999). Recent studies indicate that physical activity produces these changes by altering genes involved in synaptic plasticity (Cotman and Engesser-Cesar, 2002; Dishman et al., 2006; Farmer et al., 2004). These improvements are associated with factors that are produced within the brain or outside the brain, including BDNF and insulin-like growth factor (IGF)-1 (Carro et al., 2000). Although these studies provide strong support that physical activity beneficially alters cognitive function through alterations in plasticity related genes, it is not known whether exercise produces similar changes in gene expression in regions of the brain that control SNS activity (Mueller, 2007).

14.6 Exercise-Induced Corticospinal Plasticity

Exercise including skill, strength and endurance training induces experience-specific patterns of plasticity across motor cortex and spinal cord. Acquired motor behaviors also endure in the absence of continued training, demonstrating

that motor experience is somehow persistently encoded within the nervous system. There is extensive evidence that motor training can induce structural and functional adaptation ("plasticity") within several motor areas, including basal ganglia (Conner et al., 2003; Graybiel, 2005), cerebellum (De Zeeuw and Yeo, 2005), and red nucleus (Hermer-Vazquez et al., 2004). Virtually all acquired motor behaviors contain some element of skill learning, increased strength, and increased endurance that develop through extensive practice. In this article, skill training is defined as the acquisition and subsequent refinement of novel combinations of movement sequences. Strength training is defined as resistance excise resulting in an increase in force capacity, and endurance training is defined as exercises that increase the capacity for continued motor output.

14.6.1 Skill Training

Primary motor cortex is organized into highly interconnected neural assemblies that control discrete movements across different joints. The coordinated activation of these assemblies then encodes complex, multijoint movements. It has been proposed that the acquisition and refinement of novel movements' sequences during skill learning involve changes in the connectivity between these neural assemblies (Monfils et al., 2005). These changes are reflected as alterations in cortical synapse number, synaptic strength, and the topography of stimulation-evoked movement representations.

Adults rats trained on a complex visuomotor task that requires the animals to learn limb placement and posture control to traverse a complex obstacle course show increases in synapse number within motor cortex compared with control animals that walked an unobstructed course (Jones et al., 1999). Similarly, skilled reach training increases the complexity and density of forelimb motor cortical dendritic processes (Allred and Jones, 2004) and synapses per neuron (Kleim et al., 2004). The increase in synapse number is consistent with enhanced postsynaptic potentials within the hemisphere contralateral to the trained paw.

These synaptic changes are thought to represent changes in cortical circuitry resulting in a reorganization of the neural assemblies encoding movement. In support of this notion, reach-trained rats show an expansion of microstimulation-evoked movement representations within motor cortex corresponding to the specific motor region that controls the muscle groups used in the task. The specificity of the training induced cortical reorganization was demonstrated in squirrel monkeys initially trained on a skilled digit manipulation task that induced only an expansion of digit representations in motor cortex. Cessation of skilled digit training followed by training on a skilled wrist manipulation task reduced the previously enlarged digit representational map while also expanding the wrist motor map in the same animals (Nudo et al., 1996). Skilled

reach-trained rats also exhibit an expansion of wrist and digit movement representations in the motor cortex compared with controls. Furthermore, this learning induces synaptogenesis that is colocalized to regions in motor cortex exhibiting the expansion of wrist and digit cortical map reorganization. The reorganization of motor maps is not simply due to increased forelimb use, as demonstrated by the lack of distal forelimb motor map expansion in rats trained to repetitively press a lever (Kleim et al., 2003). Similarly, squirrel monkeys trained to retrieve food pellets from a large well that does not require the development of novel skilled digit movements do not show any change in digit movement representations despite having produced up to 13,000 digit flexions during the course of training (Plautz et al., 2000). These data indicate that training on a novel skill produces alteration in the neural circuitry in the motor cortex that are specific to the muscle groups necessary for execution of the trained task and do not occur following simple repetitive use of those same muscle groups.

Transcranial magnetic stimulation and neuroimaging techniques have been used to demonstrate similar changes in human motor cortex. Subjects trained to produce skilled digit movements on a piano show an increase in the area of digit representation corresponding to the trained hand and a decrease in motor-evoked potential (MEP) threshold (Pascual-Leone et al., 1995). These changes do not occur in the contralateral, nonplaying hand or in control subjects. Training subjects on skilled ankle (Perez et al., 2004) or tongue (Svensson et al., 2003) tasks also increases movement representation area and MEP amplitude compared with untrained controls. Cross-sectional studies have revealed comparable changes among individuals with varying degrees of motor skill. For example, highly skilled racket players have larger hand motor representation and enhanced MEP amplitudes compared with less proficient players and nonplaying controls (Pearce et al., 2000). Highly skilled volleyball players have significantly larger and more overlapping representations of medial deltoid and carpi radialis muscles than runners (Tyc et al., 2005). This is consistent with the fact that volleyball players have acquired coordinated shoulder movement sequences, whereas runners do not. Thus the specific nature of the reorganization is dependent on the specific behavioral demands of the training experience.

The cellular mechanisms of learning-dependent motor cortex plasticity are also being revealed. For example, skilled motor performance, motor map reorganization, and synapse number are all dependent on constitutive protein synthesis within motor cortex (Kleim et al., 2003). Although the specific proteins required for such plasticity are yet to be identified, BDNF appears to be involved. Inhibition of BDNF by injection of antisense oligodeoxynucleotides, receptor antagonists or BDNF receptor antibodies in rat motor cortex disrupts motor reorganization and impairs performance of motor skill (VandenBerg et al., 2004). Experimental data demonstrate that increases in protein synthesis, synaptogenesis, and map reorganization within motor cortex represent a set of

coordinated neuronal changes that drive the acquisition and performance of skilled movement.

Studies of skill training have traditionally focused on supraspinal areas and have largely ignored the spinal cord. Despite its central role as the final common pathway for motor behavior, the spinal cord's contributions to motor skill remain largely unknown. However, operant conditioning of the spinal reflexes provides some insight into how learning can induce spinal cord plasticity. The spinal stretch reflex (SSR) occurs in response to a sudden muscle stretch that is detected by muscle spindles whose afferents synapse with α-motoneurons that then synapse with the muscle. The H reflex is the electrical analog of the SSR that is elicited by electrically stimulating the 1a afferents. The increase or decrease changes of the SSR or the H reflex persist after spinal cord transection, demonstrating that the adaptation is within the cord itself (Wolpaw and Lee, 1989). The conditioned decrease in the H reflex is due to an increase in motoneuron firing threshold, wheres a reflex increase is due to reduced inhibition of the motoneuron. Decreasing the H reflex causes an increase in GABAergic synapses onto spinal cord and reduced motoneuron axonconductance. These changes likely reflect increased inhibition, in animals conditioned to decrease H reflex, from interneurons driven by corticospinal afferents.

Changes in spinal reflexes can also be observed during long-term training of more complex limb movements. People trained to walk backward for several weeks show progressive changes in the H reflex elicited at different points in the step cycle (Ung et al., 2005). Over several weeks of training, the H reflex elicited during the stance and late-swing phases of backward locomotion is decreased without any change in soleus MEPs evoked by TMS. These data demonstrate that motor skills, at least those that are operant conditioned, induce alterations in the spinal cord. Further studies using different skilled training paradigms are necessary before the exact nature of learning-induced changes in the spinal cord will be understood.

14.6.2 Strength Training

Although strength training induces well-characterized intramuscular adaptations, growth of muscle fibers cannot account for all observed increases in strength. The central nervous system contributes to training-dependent increases in strength. However, it is unclear whether and to what extent strength training induces predominately cortical versus spinal cord plasticity. Comparing TMS- and transcranial electrical stimulation (TES)-evoked MEPs can provide a means of separating out activity-dependent changes that are either predominately cortical or corticospinal. TMS primarily activates corticospinal neurons via indirect activation of transynaptic cortical neurons and TES stimulates the corticospinal tract monosynaptically. However, the slope of the relationship between MEP amplitude and torque was smaller during

submaximal contractions suggesting plasticity within spinal cord circuits. In addition, strength-trained individuals display lower TMS-evoked MEPs relative to untrained controls, suggesting a training induced decrease in corticospinal excitability (Carroll et al., 2002; Jensen et al., 2005).

Changes in spinal cord circuitry, as evidenced by modifications in reflex physiology, have also been observed in association with resistance training. Wheres the H reflex is used to assess the excitability of spinal α-motoneurons and the synaptic efficacy of 1a afferents, the V-wave measures reflect the overall magnitude of efferent motor output from the α-motoneuron pool, resulting from descending central pathways. Thus the H reflex is thought to reflect spinal excitability, wheres the V wave reflects corticospinal drive. Cross-sectional studies have demonstrated H reflex excitability is reduced wheres the V wave is increased in power-trained athletes compared with both endurance athletes and controls (Maffiuletti et al., 2001; Sale et al., 1983). Thus power training decreases the relative number of motoneurons activated by the 1a afferent volley. However, the increased V wave indicates enhanced corticospinal input in strength-trained athletes and reflex responses may then be altered during voluntary contraction. Indeed, several weeks of strength training cause significant increases in H-reflex and V-wave amplitudes when the measurements were taken during participants' maximum voluntary contractions. This suggests that strength training may be associated with increased motoneuron excitability (Aagaard et al., 2002). However, strength training also leads to decreases in TMS-evoked MEPs. Thus the increased excitability does not appear to be mediated by enhancement of corticospinal projections as measured by TMS. However, the descending output elicited by TMS of the motor cortex certainly differs substantially from the output generated physiologically to produce voluntary movement. Strength training may lead to an enhanced capacity for motoneuron recruitment and sustained activation that is not detectable by single-pulse TMS. Skill training induces reorganization of movement representations, wheres strength training alters spinal motoneuron excitability and induces synaptogenesis within spinal cord but does not alter motor map organization (Remple et al., 2001).

14.6.3 Endurance Training

In motor cortex, the primary effect of endurance training is on cerebrovasculature. Excise induces angiogenesis and increases blood flow, presumably to meet the increased metabolic demands of cortical neurons. Animals given free access to a running wheel for 30 days had increased blood flow and greater angiogenesis compared with sedentary rats. Exercise-induced changes in blood flow and angiogenesis were specific to the motor cortex and were not found in the frontal cortex or other subcortical areas, indicating that these changes are specific to areas activated by the training (Swain et al., 2003). Furthermore,

30 days of training are sufficient to induce angiogenesis within motor cortex but it does not alter motor map topography (Kleim et al., 2002). Despite the lack of exercise-induced quantifiable increases in synaptogenesis in the motor cortex, exercise training does upregulate neurotrophic factors that promote neuronal survival and differentiation (Vaynman, 2005). Moderate motor activity, such as animals running on an unobstructed walkway for several minutes a day and voluntary exercise in a running wheel, elevates the expression of BDNF in the motor cortex. However, wheres skilled training induced both increases in BDNF levels and tyrosine kinase receptors, excise only affected BDNF levels (Klintsova et al., 2004). These data indicate that it is possible that exercise may act to create a more supportive and nutritive neural environment, possibly through increased vasculature, blood flow, or growth factors, but that it does not induce any significant changes in cortical circuitry.

As with strength and skill training, the majority of studies examining changes in spinal cord circuitry with endurance training have examined changes in spinal reflexes. Endurance-trained subjects have enhanced H, Achilles tendon and patellar tendon reflexes (Koceja et al., 2004). The size of the soleus H reflex also increases as a function of daily activity. It is larger in moderately active people than in sedentary people and even larger in extremely active people. However, the H reflex is smaller in ballet dancers than sedentary people (Nielsen et al., 1993). This finding indicates the difficulty in distinguishing between the different forms of motor training. Ballet dancing requires enhanced endurance, strength, and skill. Thus smaller H reflexes likely reflect the combination of these other forms of motor training. Because cocontraction of muscles is associated with greater presynaptic inhibition, the persistent cocontractions essential for skilled movement, such as in ballet dancing, may lead to lasting decrease in transmission at the 1a synapse and thus explain the reduction in H reflex. Such changes might enhance the precision of individual movements. Thus activity alone is not the sole signal for driving spinal cord plasticity. Plasticity is dependent on the demands of the task rather than simply the amount of motor activity.

14.6.4 Plasticity of Chronic Corticospinal Injuries

Environmental enrichment, including social interactions and exercise, induces plasticity in the brain of normal animals. Environmental enrichment also produces improved outcome after neural injury. The spinal cord is capable of extensive plasticity independent of the brain and that plasticity in the brain might greatly influence spinal cord function after injury. In addition, the chronically injured spinal cord is capable of recovery after exposure to an enriched environment (Fischer and Peduzzi, 2007). The combination of physical activity and social interaction was superior to either social interaction or physical activity alone after cerebral infarction. Exercise or enriched

environment after acute spinal cord injury produced similar functional improvements. However, having similar functional improvement does not necessarily mean that the same mechanism is involved. Both running and the enriched environment in C57BL/6 mice led to a similar number of newly generated neurons 1 month after treatment but through different mechanisms. Running increased the initial proliferation, and enrichment increased cell survival (van Praag et al., 1999). These studies may also have implications in the rehabilitation of people with chronically injured spinal cords. Intensive training and exercise may enhance motor recovery and functional improvement can occur in people who have been long paralyzed due to spinal cord injury.

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Part IV
Mechanosensitivity of Neurovascular System

Chapter 15
Mechanosensitive Calcium Fluxes in the Neurovascular Unit: TRP Channel Regulation of the Blood-Brain Barrier

Rachel C. Brown and Roger G. O'Neil

Abstract The blood-brain barrier of the neurovascular unit is a critical organ for normal brain function. It is continuously exposed to mechanical stress both from the peripheral circulation and plasma osmolarity changes. Barrier integrity is regulated by a number of mechanisms, including influx of calcium ions and activation of calcium-sensitive signaling pathways. This review addresses the molecular identity of the channels underlying mechanosensitive calcium influx, and hypothesizes a central role for transient receptor potential channels in mechanosensitive regulation of blood-brain barrier endothelial cell function.

Keywords Calcium entry pathways · mechanically gated channels · mechanosensitive channels · voltage-gated channels · purinergic receptors · sodium-calcium exchanger · transient receptor potential channels · calcium-dependent modulation

15.1 Introduction

The neurovascular unit (NVU) is a critical feature of the brain circulatory system, and regulates normal cerebral blood flow and brain function. The NVU (Fig. 15.1) consists of multiple cell types, including endothelial cells, astrocytes, pericytes and neurons (Abbott et al., 2006). In brain capillaries, the NVU is characterized by the presence of the blood-brain barrier (BBB), a structure consisting of tight junctions between capillary endothelial cells (Rubin and Staddon, 1999) and the polarized expression of specific transport systems (Dermietzel and Krause, 1991). The tight junctions between brain capillary endothelial cells are sufficient to impart high electrical resistances across the capillary wall (Rubin and Staddon, 1999), indicating that paracellular diffusion of ions from the blood to the brain is highly restricted. The BBB is critical in

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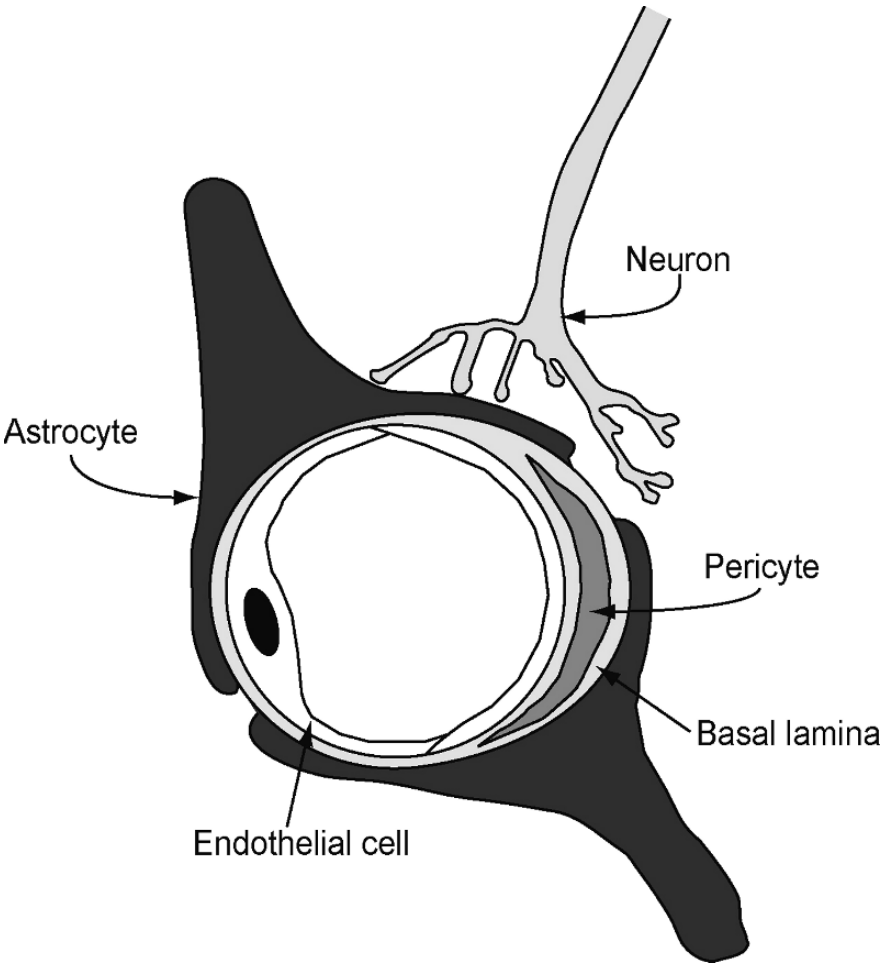

Fig. 15.1 The neurovascular unit and blood-brain barrier. The neurovascular unit consists of multiple cell types. Endothelial cells that form the walls of the brain capillaries, surrounded by the basal lamina, form the walls of the brain capillaries. Astrocytes send foot processes to the capillaries, which wrap around the basal lamina along portions of the capillary. Neuronal processes also extend to the capillaries. Finally, pericytes may be found within the basal lamina, where they can interact with capillary endothelial cells

regulating macromolecular and ionic gradients within the brain, allowing for normal neuronal activity.

The integrity of the NVU/BBB is compromised in numerous pathological conditions, including Alzheimer's disease, multiple sclerosis, inflammation, diabetes and stroke (Ballabh et al., 2004; Hawkins and Davis, 2005). These disruptions allow for the diffusion of proteins, ions, water and other compounds from the blood into the brain. Many disruptive stimuli share a common

cellular event within the endothelial cells: an increase in the level of intracellular calcium and the activation of calcium signaling cascades (Aschner et al., 1997; Nagashima et al., 1997; Bartha et al., 2000; Brown and Davis, 2002; Easton and Abbott, 2002). Prevention of calcium influx can protect the BBB in a number of scenarios (Abbruscato and Davis, 1999; Easton and Abbott, 2002), implicating calcium ions as critical regulators of BBB structure and function (Brown and Davis, 2002).

For some disrupting stimuli, such as stroke, the dysregulation of plasma macromolecule and ion fluxes across the capillary walls in the infracted area contributes to the osmotic movement of water into the brain, resulting in edema formation (Petty and Wettstein, 2001). This edema contributes to secondary injury to neurons. While many studies have addressed the role of hypoxic stress in disrupting the BBB (Plateel et al., 1997; Abbruscato and Davis, 1999; Fischer et al., 2002; Mark and Davis, 2002; Brown et al., 2003; Fischer et al., 2004; Mark et al., 2004; Krizbai et al., 2005), the role of mechanical stress associated with a loss or reduction in blood flow and shear stress as a potential contributing factor to NVU endothelial cell dysfunction in stroke is not fully appreciated. In this review we will discuss the potential role of mechanical stresses and their alteration in the regulation of the NVU, particularly the altered mechanical stress experienced by NVU endothelial cells in response to changes in blood flow and blood osmolarity.

15.2 Calcium Entry Pathways: A Mechanosensitive Link

As previously mentioned, many stimuli that disrupt the BBB cause an increase in intracellular calcium, including thrombin (Lerner, 1994; Bartha et al., 2000; Kim et al., 2004), histamine (Li et al., 1999; Paemeleire et al., 1999; Paltauf-Doburzynska et al., 2000), bradykinin (Paemeleire et al., 1999; Easton and Abbott, 2002), ATP (Tanaka et al., 2004), and exposure to hypoxic stress (Kimura et al., 2000; Brown et al., 2004b). Blocking elevation of intracellular calcium prevents BBB disruption by many of these treatments (Li et al., 1999; Bartha et al., 2000; Brown et al., 2004b; Tanaka et al., 2004; Brown et al., 2008). However, the underlying mechanisms of calcium entry, and the subsequent events in NVU endothelial cells, are not well understood, especially for mechanical stimuli.

15.2.1 Voltage-Gated Channels

There are a number of potential protein candidates for calcium entry channels in NVU endothelial cells. Pharmacological evidence exists for the presence of L-type calcium channels in these cells (Abbruscato and Davis, 1999; Berkels et al., 1999; Yakubu and Leffler, 2002), with L-type calcium channel blockers

protecting against barrier disruption. However, there is little functional evidence for voltage-gated calcium channels in these non-excitable cells, and no convincing molecular evidence for the expression of L-type calcium channels in NVU endothelial cells. Furthermore, there is evidence of off-target effects of L-type channel blockers on calcium fluxes (Berkels et al., 1999; Hempel et al., 1999), and they are not effective at preventing intracellular calcium increases or barrier disruption (Stanimirovic et al., 1994; Ikeda et al., 1997; Wei et al., 2004). Hence a role for voltage-gated channels in NVU mechanosensitive phenomena is not anticipated. However, L-type calcium channels are present on the other cells of the NVU, including astrocytes, pericytes and neurons (Barres et al., 1989; Catterall, 1998; Westenbroek et al., 1998; Kamouchi et al., 2004), and may be important in mediating calcium influx in those cell types.

15.2.2 Purinergic Receptors

Purinergic receptors are expressed on NVU endothelial cells, specifically P2Y receptors (Nobles et al., 1995; Sipos et al., 2000). Other endothelial cells express P2U receptors (Miyagi et al., 1996) and P2X receptors (Ramirez and Kunze, 2002; Harrington et al., 2007), but these have not been found at the BBB. P2Y receptors are G protein-coupled receptors (GPCR) (Burnstock, 2006), while P2X receptors are calcium-permeable ligand-gated ion channels. Activation of P2Y receptors increases intracellular calcium levels in endothelial cells by inducing release of calcium from intracellular storage sites (Albert et al., 1997; Duchene and Takeda, 1997), and can mediate alterations in vascular endothelial cell monolayer permeability (Tanaka et al., 2004). However, since P2Y receptors are GPCR, these signaling receptors do not directly mediate calcium influx at the BBB.

15.2.3 The Sodium-Calcium Exchanger

A striking characteristic of NVU endothelial cells is the high level of polarized expression of numerous transporters, which allow for CNS penetration of ions, peptides and drugs (Keep et al., 1993; O'Donnell et al., 1995; Vannucci et al., 1997; Jolliet-Riant and Tillement, 1999; Chishty et al., 2003). A major transporter involved in calcium flux at the BBB is the sodium-calcium exchanger (NCX) (Sedova and Blatter, 1999). Under normal physiological conditions, the NCX transports calcium out of the cells in exchange for sodium, maintaining a low intracellular calcium concentration. However, under stroke-like conditions, with elevated intracellular sodium levels, the NCX can reverse (Li et al., 2000; Berna et al., 2001), potentially contributing to calcium influx and activation of calcium signaling cascades and subsequent downstream cellular responses.

15.2.4 Transient Receptor Potential Channels

Recent studies in our laboratory have focused on the transient receptor potential (TRP) channel superfamily as candidates for mediating calcium influx in NVU endothelial cells. There are currently seven TRP families, and the channels can be activated by many different stimuli (Venkatachalam and Montell, 2007). Several of these families encode cation permeable channels that mediate calcium flux in a number of cell types (Caterina et al., 1997; Pizzo et al., 2001; Gao et al., 2003; Nilius et al., 2003; Kraft et al., 2004; White et al., 2006). A number of mammalian TRP channels are characterized as "mechanosensitive", including TRPA1, TRPP, TRPC1, TRPC6, TRPV1, TRPV2 and TRPV4 (Nilius and Voets, 2004; Lin and Corey, 2005; O'Neil and Heller, 2005; Spassova et al., 2006)

The TRPA1 channel is located on the stereocilia in hair cells of the mouse inner ear. siRNA downregulation of protein expression reduces transduction currents in transfected hair cells (Corey et al., 2004). Embryonic expression of TRPA1 is correlated with the onset of mechanosensitivity in hair cells (Corey et al., 2004). The multiple ankyrin repeats of TRPA1 are hypothesized to act as a gating spring, transducing stereocilia bending to channel opening (Sotomayor et al., 2005). However, there is no evidence for TRPA1 expression in vascular endothelial cells in general (Yao and Garland, 2005), or in NVU endothelial cells specifically, although to date the presence of this, and many other TRP channels has not been specifically examined at the BBB.

TRPP channels are associated with polycystic kidney disease (Delmas, 2005). Two proteins in the TRPP family, PKD1 (TRPP1) and PKD2 (TRPP2), have been linked to mechanosensation in primary cilia of kidney tubule epithelial cells (Nauli et al., 2003). Expression of both proteins is required for the expression of functional ion channels (Hanaoka et al., 2000), with TRPP2 being the pore-forming subunit. Knockout of PKD1 eliminates flow-activated calcium influx in kidney epithelial cells (Nauli et al., 2003), indicating activation of the channel by fluid flow/shear stress. PKD1 has been proposed as the flow sensor in the TRPP mechanosensitive channel (Forman et al., 2005). Activation of the channel is thought to occur by actual mechanical deformation of the extracellular domains of PKD1 by fluid flow through the kidney tubule (Forman et al., 2005), and presumably in other TRPP expressing tissues. Like TRPA1, the presence of TRPP channels at the BBB has not yet been described.

TRPC1 has been implicated in mechanosensation to stretch in oocytes (Maroto et al., 2005), but does not appear to be responsible for smooth muscle activation after hypo-osmotic swelling or increases in pressure (Dietrich et al., 2007), indicating that TRPC1 alone may not serve as a mechanosensitive channel in endogenous expression systems. Mechanisms of activation for TRPC1 include store-depletion, (Beech, 2005), and stretch, though the exact process by which stretch can activate TRPC1 is unclear. More recent studies on TRPC1 and TRPC6 have indicated that these channels may not underlie mammalian stretch-activated mechano-sensitive Ca^{++} permeable cation channel

(MscCa) activity (Gottlieb et al., 2007). Our recent work has demonstrated expression of TRPC1 in NVU endothelial cells (Brown et al., 2008), as well as TRPC2, C4 and C7, none of which are implicated in mechanosensation at this time. There is some evidence for the expression of TRPC6 in the NVU (Brown et al., 2008), but it is not clear if this expression is in the endothelial cells or in other cell types.

The TRPV channels are strongly implicated in the transduction of mechanical stress into cellular responses (O'Neil and Heller, 2005). TRPV4 was first identified in the kidney tubule, and mediates responses to hypo-osmolar treatment (Liedtke et al., 2000) and shear stress (Gao et al., 2003; Taniguchi et al., 2007; Wu et al., 2007). TRPV4 knockout mice show impaired responses to heat, osmolarity and pressure (Liedtke and Friedman, 2003; Mizuno et al., 2003; Suzuki et al., 2003; Lee et al., 2005; Levine and Alessandri-Haber, 2007), indicating that TRPV4 serves as an important member of a mechanosensitive complex. TRPV1 and TRPV2 are also implicated in transducing mechanically-induced signals (Birder et al., 2002; Muraki et al., 2003). Our data indicates that NVU endothelial cells express TRPV2 and TRPV4 (Brown et al., 2008), both of which potentially mediate mechanical signal transduction at the BBB.

In summary, although there are a number of transport proteins that might mediate calcium influx into NVU endothelial cells, the TRP channels are the best candidates for transducing mechanical signals. Of the multiple TRP isoforms that are mechanosensitive, expression of TRPC1, TRPV2 and TRPV4 has been demonstrated at the BBB (Brown et al., 2008). In particular, since TRPV4 has been shown to be sensitive to hypotonic cell swelling in aortic endothelial cells (Vriens et al., 2005), to flow in mid-cerebral artery endothelial cells (Kohler et al., 2006; Marrelli et al., 2007) and to both flow and hypotonic cell swelling in renal collecting cells (Gao et al., 2003; Wu et al., 2007), it is likely that the channel also plays a significant role in mechanosensitive properties of the BBB.

15.3 Calcium-Dependent Modulation of BBB Integrity

A common event in the disruption of the BBB by various stimuli is an increase in intracellular calcium (Greenwood, 1991; Nagashima et al., 1997; Abbruscato and Davis, 1999; Paemeleire et al., 1999; Bartha et al., 2000), leading to subsequent loosening of BBB tight junctions and increased barrier permeability. We hypothesize two mechanisms by which calcium ions regulate barrier function: (1) interaction of calcium ions with the proteins of tight and adherens junctions, either intracellularly at the site of the junctional complex, or in the extracellular space (Fig. 15.2), and (2) modulation of calcium-sensitive signaling pathways that can alter protein phosphorylation, cell-cell adhesion and barrier function.

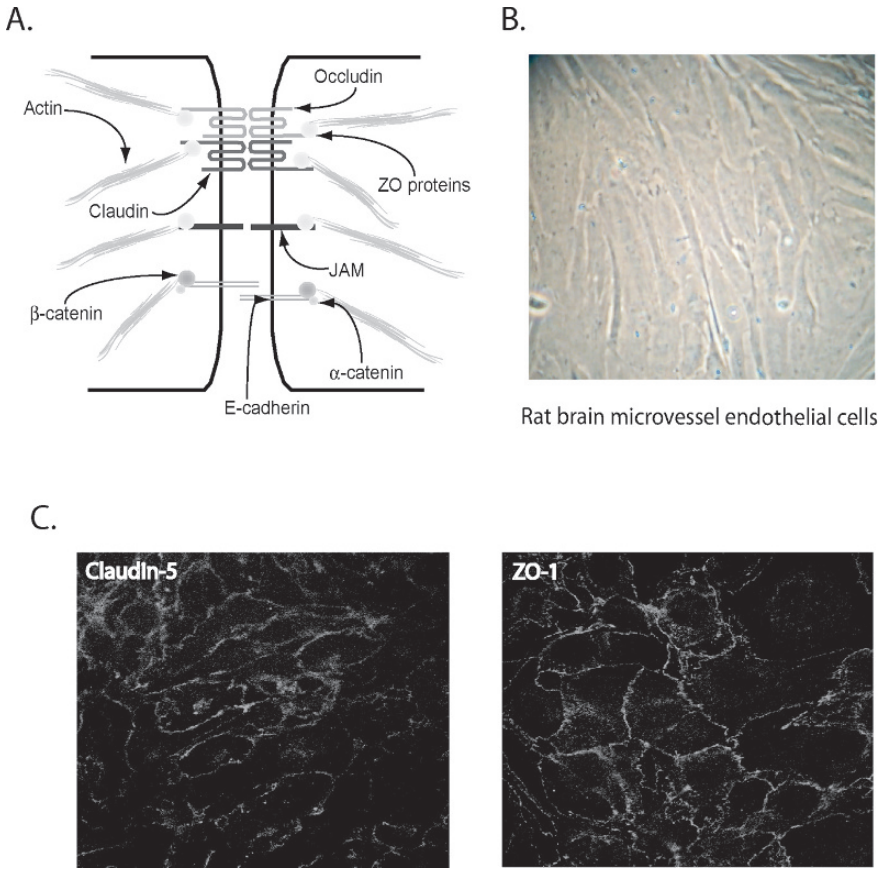

Fig. 15.2 Tight junctions in BBB endothelial cells.
The tightness of the BBB is due to the presence of tight junctions between adjacent endothelial cells. (**A**) The tight junction, at the apical or blood side of the brain capillary endothelial cell, consists of multiple transmembrane proteins (occludin and members of the claudin family) that are anchored to the actin cytoskeleton by the zonula occludens (ZO) proteins. Adherens junctions are present towards the basolateral, or brain, side of the capillary wall. Another contributor to cell-cell adhesion is the JAM family of proteins. (**B**) Rat brain microvessel endothelial cells in culture form characteristic spindle shapes and are closely packed. (**C**) Confocal microscopy staining for two tight junction proteins in an immortalized mouse brain endothelial cell line. Both claudin-5 and ZO-1 are closely aligned with the cell membrane, indicating their localization in tight junctions

15.3.1 Protein-Calcium Ion Interactions

There is certainly evidence of a direct role for calcium in the maintenance of adherens junctions between adjacent cells (Gumbiner and Simons, 1987; Gumbiner et al., 1988; Bazzoni and Dejana, 2004), but this is directly linked to

extracellular, not intracellular calcium levels. Chronic depletion of extracellular calcium can also disrupt localization of zonula occludens-1 (ZO-1) in the tight junction (Riesen et al., 2002), but the time course of this response does not allow for differentiating between direct ion interactions and signaling cascade actions of increased intracellular calcium. There is evidence that low intracellular calcium levels interfere with tight junction assembly and function (Stuart et al., 1994; Ye et al., 1999), but again, there is no evidence that this is due to direct interactions of calcium ions with tight junction proteins. To date, the literature does not suggest a direct role for calcium in regulating BBB tight junctions and barrier function, such as that seen with adherens junctions. However, modulation of adherens junctions can also contribute to BBB leakiness (Tiruppathi et al., 2002).

15.3.2 Calcium-Dependent Signaling Pathways

While a direct role for calcium in modulating BBB function is not currently supported by the literature, there is ample evidence for an indirect role for calcium in mediating barrier function via calcium sensitive signaling cascades. Endothelial barrier disruption by inflammatory mediators, such as thrombin and histamine, can occur via calcium-induced activation of myosin light chain kinase (MLCK) and dissociation of adherens junctions (Tiruppathi et al., 2002). Studies in lung endothelial barriers indicate that thrombin exerts its disruptive effects via calcium-calmodulin kinase II (CaMKII) and the ERK signaling pathway (Borbiev et al., 2001; Borbiev et al., 2003). This leads to subsequent activation of MLCK and contraction of the cytoskeleton (Gunduz et al., 2003), pulling adjacent endothelial cells apart. This signaling pathway also involves G_o linked GPCRs (Vanhauwe et al., 2002) and phospholipase C (Kim et al., 2004). Thrombin-induced calcium influx has been linked to TRPC1 (Paria et al., 2004), and thrombin-induced phosphorylation of TRPC1 via protein kinase C is important for TRPC1 activation and calcium influx (Ahmmed et al., 2004).

While the CaMKII-ERK-MLCK pathway for barrier disruption has been demonstrated extensively in endothelial cell systems, it remains to be seen if these players mediate BBB function in a similar fashion. Activation of MLCK could explain the morphological changes seen in NVU endothelial cells after exposure to numerous disrupting stimuli, but this has not been extensively studied as of yet (Haorah et al., 2005). Furthermore, there are other calcium-sensitive signaling mechanisms that may contribute to BBB disruption, including calcium-calmodulin regulated production of nitric oxide (Busse and Mulsch, 1990; Thiel and Audus, 2001; Mark et al., 2004), activation of PKC regulated signaling pathways (Grammas et al., 1998; Fischer et al., 2004; Fleegal et al., 2005), and calcium-regulated phosphorylation of tight junction proteins (Andreeva et al., 2001; Ishizaki et al., 2003; Kale et al., 2003; Ohtake et al., 2003).

15.4 Sensing Mechanical Stress at the NVU

Endothelial cells of the vasculature are highly sensitive to mechanical forces relating to pressure/stretch (biaxial stress) and to shear stress arising from blood flow (Nilius et al., 2003; Busse and Fleming, 2006). Accumulating evidence points to a likely role of TRP channels in transducing mechanical signals in NVU endothelial cells, although our understanding of the extent of the stresses, and the sensing and transduction of these stimuli into cellular responses at the NVU is still forthcoming. There are a number of pathological scenarios in which this signaling may play a role in normal CNS function. Two of the most common scenarios are discussed below: changes in shear stress/blood flow and/or blood pressure due to stroke or hypertension, and changes in plasma osmolarity, leading to cell volume changes and cell membrane stress, as occurs in hyponatremia (hypo-tonicity) or induced hyperosmolar BBB disruption to treat brain tumors. The glycocalyx appears to play a central role in sensing mechanical stresses in vascular systems and will be considered first.

15.4.1 Role of the Glycocalyx in Sensing Mechanical Stress

Any discussion of endothelial cell response to mechanical stress must include consideration of the glycocalyx. The glycocalyx is a thin network of glycoproteins lining all blood vessels. It consists of membrane-bound molecules, including sulfated proteoglycans, hyaluronan, glycoproteins and plasma proteins (Weinbaum et al., 2007). The endothelial glycocalyx is negatively charged, and typically includes glycoproteins with acidic oligosaccharides and sialic acid, as well as proteoglycans with glycosaminoglycan side chains, including heparan sulfate, chondroitin sulfate and hyaluronan (Tarbell and Pahakis, 2006; Weinbaum et al., 2007). The glycocalyx is critical to the transduction of shear stress forces to the endothelial cells, and their subsequent response to this stress. Current theories indicate that the glycocalyx mediates shear stress by transducing flow to the endothelial cells via the membrane-bound glycocalyx proteins (Tarbell and Pahakis, 2006); the endothelial cell membrane may not experience shear stress directly. The structure and function of the glycocalyx is dependent on hydration (Weinbaum et al., 2007), and can be disrupted by inflammatory mediators (Henry and Duling, 2000) or by enzymatic digestion (Vogel et al., 2000).

There have been a number of studies examining the BBB glycocalyx and its composition. Initial studies indicated no difference in glycocalyx composition between brain, retina and myocardium (Lawrenson et al., 2000), or between in vivo vessels and in vitro cultures (Fatehi et al., 1987). Removing the glycocalyx with heparinase can increase perfusion and cerebral blood flow (Vogel et al., 2000). Because of the net negative charge of the

glycocalyx, permeability of the BBB to anionic compounds is lower then the permeability to neutral compounds, particularly at smaller sizes (Sahagun et al., 1990).

An emerging model for transduction of shear stress through the glycocalyx is dependent on membrane-bound glycoproteins, including CD44 and syndecans, which transmit shear stress through the glycocalyx layer to the cell membrane (Weinbaum et al., 2007). These transducing molecules are linked to the cytoskeleton, and movement of the outer portions of the proteins in response to blood flow is conducted to the cytoskeleton through the membrane glycoproteins. The plasma membrane of the endothelial cells may not experience any shear stress directly. The subsequent consequences of the transmission of the shear stress are not well understood, but may include activation of mechanosensitive ion channels in the cell membrane, especially glycosylated channels, where the glycosylated moieties may protrude into and be a part of the glycocalyx structure.

15.4.2 Sensing Changes in Shear Stress/Blood Flow at the NVU

Shear stress attributed to blood flow has great effects on NVU endothelial cells. In culture, brain endothelial cells tend to grow in a disorganized monolayer, with no orientation to the spindle shaped cell bodies (Fig. 15.2). However, if brain endothelial cells are grown under conditions of flow, they orient parallel to the direction of the flow (Neuhaus et al., 2006) and develop higher transendothelial electrical resistance (TEER) then cells grown without flow (Stanness et al., 1997). Shear stress also induces lamellopodia formation and focal adhesion formation in endothelial cells in culture, shifting actin and vimentin stress fibers (Mott and Helmke, 2007). Shear stress inside the capillaries can change in two ways: a decrease, such as in a stroke, or an increase, as in hypertension.

15.4.2.1 Decreased Blood Flow – Stroke

Ischemia and reperfusion are known to damage the glycocalyx, altering the mechanical forces on the endothelial cells. This response can be prevented by ischemic preconditioning, and partially blocked by treatment with superoxide dismutase, indicating a role for reactive oxygen species in glycocalyx disruption (Beresewicz et al., 1998). This damage typically occurs during reperfusion, when shear stress goes from very low back to normal. The BBB is also disrupted during ischemia/reperfusion injury in vivo (Kuroiwa et al., 1988; Preston and Webster, 2002), although the endothelial cells themselves seem to recover during reoxygenation in an in vitro model (Mark et al., 2001). It is difficult to tease apart the relative contributions of hypoxic and shear stress to BBB dysfunction in animal models. An *in vivo* study by Hom and coworkers in 2001 demonstrated little or no BBB disruption with lowered perfusion pressure for a 20 min

in situ perfusion study (Hom et al., 2001). Similarly, a study of BBB endothelial cells grown in capillary flow systems demonstrated no alteration of BBB permeability after transient loss of flow for one hour (Krizanac-Bengez et al., 2003). However, there have only been a few studies on loss of flow in shear stress models, and in all of those the lowered shear stress was only for a brief period of time. Longer exposures to lowered flow may cause alterations in BBB permeability in isolation, or after reperfusion/resumption of normal shear stress in the model system.

15.4.2.2 Increased Blood Flow – Hypertension

Thanks to animal models of hypertension, there is a body of literature examining the effects of increased blood pressure on BBB structure and function. There is a well characterized decline in BBB function with age (Mooradian, 1988), which can be exacerbated by the development of high blood pressure. Several recent studies in the stroke-prone spontaneously hypertensive rat (SPSHR) and the spontaneously hypertensive rat (SHR) have investigated BBB function at increased blood pressures. SHR and SPSHR showed increased vascular permeability to horseradish peroxidase (MW 44 kDa) in the hypothalamus as compared to controls (Ueno et al., 2004). These hypertensive animals also showed disruption of the glycocalyx. High blood pressure is also correlated with increased damage after middle cerebral artery occlusion in SHR (Hom et al., 2007). These studies suggest that hypertension alone disrupts the BBB, and it exacerbates barrier opening after additional ischemic insult. This is consistent with studies in other vascular endothelial cells showing increased calcium influx with increases in shear stress (Nilius et al., 2003).

15.4.2.3 Channels Modulated by Shear Stress/Blood Flow

Stretch or shear stress applied to endothelial cells activates calcium influx as part of the cellular response. While the focus of this review is on the role of TRP channels in brain microvessel endothelial cells, other channels may also be sensitive to mechanical stimulation, including inward-rectifying potassium channels and outward-rectifying chloride channels (Gautam et al., 2006). These channels may play important roles in the vascular response, especially for the larger vessels where the interplay of endothelial cells with the surrounding smooth muscle cells may be critical. Several excellent reviews covering these other channels have been published and will not be discussed here (Coleman et al., 2004; Gautam et al., 2006).

TRP channels are emerging as potentially central players in endothelial responses to shear stress, especially in medium to large vessels (Nilius et al., 2003; Gautam et al., 2006). Several TRP channels are potential candidates for sensing shear stress/pressure in the brain microvessel endothelial cells, as discussed above. Two channels with the strongest tie-in as a sensor, or a

component of a sensor, are TRPV2 and TRPV4, both of which are expressed in brain microvessel endothelial cells (Brown et al., 2008). TRPV4 is sensitive to hypotonic cell swelling in the bEnd3 brain endothelial cell line and has been shown in separate studies to be highly sensitive to increases in shear stress in both overexpression systems and in renal epithelial cells endogenously expressing TRPV4 (Gao et al., 2003; Wu et al., 2007; Brown et al., 2008). Knockdown of TRPV4 in non-NVU cells by siRNA techniques abolishes flow- and hypotonicity-induced calcium influx (Wu et al., 2007). Further, TRPV4 has now been shown to be sensitive to fluid flow in middle cerebral artery endothelial cells (Kohler et al., 2006; Marrelli et al., 2007). Hence, it is likely that the TRPV4 channel also plays a significant role in mechanosensitive properties of the BBB, and it may be a primary source of mechanically-induced calcium influx in these cells; further supporting evidence for this and other TRP channels as mechanosensitive channels at the BBB remains to be fully established.

While TRPV4 may be sensitive to mechanical stimulation, the mechanism of activation is likely to be indirect. The kinetics of channel opening following application of shear stress are relatively slow, requiring many seconds to activate (Wu et al., 2007). Further, Nilius and coworkers demonstrated that the channel could be activated by downstream metabolites of arachidonic acid which were induced by hypotonicity-induced swelling (Vriens et al., 2004). Blockade of arachidonic acid metabolism abolished hypotonicity-induced activation of TRPV4, thereby demonstrating that the activation of TRPV4 by mechanical stimulation by an indirect biochemical pathway. Whether a similar mechanism of activation by shear stress is in play at the NVU seems highly probably, but has not yet been directly demonstrated.

15.4.3 Sensing Changes in Plasma Osmolarity and Cell Volume

Other physiological events can cause mechanical stress on NVU capillary endothelial cells, such as changes in blood osmolarity, which in turns leads to swelling or shrinking of endothelial cells. Clinically, there are a number of situations in which blood osmolarity may be altered; here we will discuss hypo-osmolarity arising from hyponatremia, and hyper-osmolarity after infusion of hyper-osmolar mannitol solutions to aid in the delivery of chemotherapeutics to brain tumors.

15.4.3.1 Hypo-Osmolarity – Hyponatremia

Hyponatremia is a condition in which plasma sodium levels drop below the normal range, due to loss of sodium or retention of water. One cause of hyponatremia in otherwise healthy people occurs in the case of endurance

athletes participating in long events, such as long distance running events (Noakes, 2002) and Ironman triathlons (Speedy et al., 1997), or with water intoxication, such as in patients suffering from psychogenic polydipsia (Dundas et al., 2007). In these cases, the patient loses sodium through sweating and urinary loss or dilutes plasma sodium levels by excessive intake of water. The loss of sodium in athletes can be exacerbated by intake of excessive amounts of water, leading to water intoxication and low plasma sodium levels (Speedy et al., 1997; Noakes, 2002; Noakes, 2003). In some instances, overhydration and hyponatremia can be fatal in otherwise healthy individuals (Garigan and Ristedt, 1999). The greatest risk to individuals with hyponatremia is the development of cerebral edema, leading to disorientation and seizures (Oster and Singer, 1999; Baker et al., 2000).

In normonatremia, blood osmolarity is typically near 290 mOsm/l, whereas hyponatremic osmolarity can drop to 250 mOsm/l (Oster and Singer, 1999). Low plasma osmolarity alone does not seem to disrupt the BBB in the clinical setting, but we have shown that hypotonicity can induce a transient disruption of the BBB *in vitro* (Brown et al., 2008). Instead, it is the rapid correction of plasma sodium levels that causes disruption of the BBB, potentially leading to osmotic demyelination syndrome (Adler et al., 1993; Adler et al., 1995; Baker et al., 2000). This rapid correction can also lead to increased cerebral blood flow (Adler et al., 2000), and allows for the penetration of the brain by inflammatory mediators such as IgG and activated complement C3d (Baker et al., 2000). Furthermore, chronic hyponatremia seems to lead to a lower osmotic threshold for BBB disruption after correction (Adler et al., 1995), indicating that the endothelial cells of the BBB adapt to lowered plasma osmolarity in some fashion that makes them more sensitive to a rapid increase in osmolarity with correction, leading to an increased risk of BBB disruption and edema formation. Whether this is related to activation of hypo-osmotic-sensitive TRP channels, such as TRPV4, is currently not known. NVU endothelial cells express two hypo-osmolar sensing channels, TRPV2 and TRPV4 (Brown et al., 2008).

15.4.3.2 Hyper-Osmolarity – Delivery of Chemotherapeutic Agents to Inoperable Brain Tumors

The ability of hyper-osmolar agents to reversibly open the BBB has been known for almost forty years (Rapoport, 1970; Rapoport et al., 1971). Infusion of hyperosmolar mannitol is used clinically to enhance the delivery of chemotherapeutic agents across the BBB to targeted tumors (Neuwelt et al., 1979; Gumerlock et al., 1992). In animal models, there is regional variation in the degree of barrier disruption in the brain (Brown et al., 2004a), and hypertension alters the time course of barrier opening (Al-Sarraf et al., 2007). Exposure to hyper-osmolar mannitol triggers calcium influx into BBB endothelial cells (Paemeleire et al., 1999) and disrupts tight junction structure (Nagy et al.,

1979). This calcium influx is thought to trigger signaling cascades that result in BBB disruption (Nagashima et al., 1994; Nagashima et al., 1997). However, BBB disruption by hyper-osmolar solutions is transient, and the barrier recovers within a short period of time (around 1 hr) (Brown et al., 2008). This rapid response and subsequent normalization would suggest an acute cellular response involving receptor activation and desensitization rather then global cellular changes involving the expression of new proteins.

The mechanism of the hyper-osmotic barrier disruption and associated calcium influx is currently not defined. Could it involve shrinkage-induced activation of TRP channels? This is an intriguing possibility since a similar scenario may be at play in the osmo-sensitive neurons of the hypothalamus that control vasopressin secretion from the posterior pituitary. Application of hyper-osmotic media to neurosecretory supraoptic nucleus neurons and to the organum vasculosum lamina terminalis neurons have been shown to induce calcium influx in these cells as part of a mammalian osmoreceptor (Ciura and Bourque, 2006; Sharif Naeini et al., 2006). These investigators demonstrated that the basis of the calcium influx may be an N-terminal splice variant of the TRPV1 channel. The variant channel is activated by cell shrinkage leading to an influx of calcium. Such a channel could underlie the effects of hyper-osmolar solutions on calcium influx and the BBB integrity of the NVU. However, it is currently not know if this splice variant of TRPV1 is expressed in the brain microvessel endothelial cells or whether splice variants of other TRPV channels may play a role in shrinkage-induced activation of calcium influx.

15.5 Conclusions

The BBB/NVU is a critical structure for CNS microenvironmental regulation and can respond to changes in mechanical stress in a number of ways. While the exact mechanism of transducing mechanical stress is not currently known, calcium-permeable channels of the TRP superfamily are attractive candidates for mediating some of this signaling. Endothelial cells of the NVU express several mechanosensitive TRP channels, including TRPC1, TRPV2 and TRPV4 (Fig. 15.3); the presence of other mechanosensitive TRP channels, such as TRPA1 or TRPP1, is currently unknown. Activation of mechanosensitive channels leads to calcium influx, triggering alterations in tight junction function and changes in BBB permeability. These TRP channels are positioned to detect changes in mechanical stress in the cerebral vasculature due to changes in shear stress/blood flow or blood pressure, as in stroke or hypertension, or changes in plasma osmolarity that can lead to alterations in endothelial cell volume. The precise regulatory mechanisms remain to be elucidated in future studies.

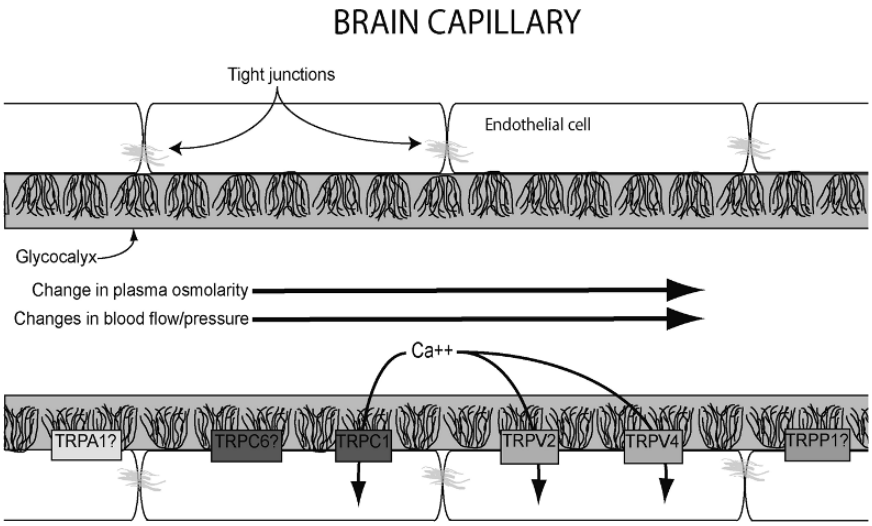

Fig. 15.3 Calcium influx trigger by mechanical stress at the blood-brain barrier. Changes in either blood flow or pressure (shear stress) or plasma osmolarity (osmolar stress) can trigger calcium influx in NVU endothelial cells through a number of the TRP family of ion channels. This calcium influx may be critical in the regulation of BBB functional integrity after alterations in mechanical stress in brain capillaries, such as loss of flow following stroke or hemorrhage

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