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Microbial Endocrinology

Mark Lyte *Editor*

Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health

Second Edition

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As always I wish to thank my family
for all their support and understanding
and especially to the Wah-wah for all his
dedication and assistance.

Preface

In the concluding section of the first chapter of the previous edition, I had written "And finally, as the oft-used cliché goes 'this is not the end of the story, just the beginning (Lyte 2010)'". The recent advances made over the last 5 years since the publication of the first edition have provided ample evidence that this has been, and hopefully will continue to be, the case. This second edition contains over 50 % new and revised content. Prominent among these has been the emergence of the microbiota–gut–brain axis and the role it plays in brain function. Microbial endocrinology, and the production of neurochemicals that the microbiota produce, provides for a mechanism (and surely *not* the sole one) by which the microbiota may influence the nervous system. In large measure, the concept of microbial endocrinology has been viewed by others as a "one-way street" in that it was usually the host's production of neurochemicals (and in the main stress-related ones) that formed the early basis for the development of the theory. The realization that metabolite production by microbiota has now been shown by groups to include many of the biogenic amines (such as the catecholamines, serotonin, and histamine) all of which are produced in quantities sufficient to impact host neurophysiology (Asano et al. 2012; Sridharan et al. 2014). As such, the other direction of this "two-way" street that has always been part of the microbial endocrinology theory to include effects of the microbiota on the host due to microbiota-derived neurochemical production is now beginning to be explored. The next few years will be, as they are also wont to say, "highly interesting". The journey continues …

Ames, IA, USA — Mark Lyte
September, 2015

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Chapter 1
Microbial Endocrinology: An Ongoing Personal Journey

Mark Lyte

Abstract The development of microbial endocrinology is covered from a decidedly personal perspective. Specific focus is given to the role of microbial endocrinology in the evolutionary symbiosis between man and microbe as it relates to both health and disease. Since the first edition of this book series 5 years ago, the role of microbial endocrinology in the microbiota-gut-brain axis is additionally discussed. Future avenues of research are suggested.

Keywords Neurochemicals • Stress • Neurophysiology • Microbiota-gut-brain • Probiotics • Microbiology

1.1 Introduction

The development of the field of microbial endocrinology has now spanned 23 years from the time I first proposed its creation in 1992 (Lyte 1992; Lyte and Ernst 1992). During that time, this interdisciplinary field has experienced two of the characteristics of a typical microbial growth curve: a long lag phase during which acceptance of articles was problematic to say the least, followed by an early log phase of growth characterized by increasing awareness that the intersection of microbiology, endocrinology and neurophysiology offers a unique way to understand the mechanisms underlying health and disease. This book, I am happy to report, comes at the start of that early log phase with the possibilities for future rapid growth.

As is more common than many are often wont to admit, the development of a new discipline does not occur in a vacuum and if one chooses to look hard enough, one can usually find reports dating back many decades, which document many of the experimental findings that help form the founding tenets of the new discipline.

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Given the history of use of neuroactive substances in the treatment of human disease, which provided for ample opportunity for the interaction of microorganisms with neuroendocrine hormones, it is not surprising that such is the case in microbial endocrinology (Lyte 2004). It is a testament to how prevailing notions of what separates "us" from "them" can influence scientific inquiry that scientists of a bygone era did not fully recognize that the ability of a lowly bacterium to both produce and recognize substances that are more commonly thought of as defining a mammal (i.e. vertebrate nervous system) could prove critical to both health and disease. Indeed, J.A. Shapiro may have put it best by titling, in part, his article covering his nearly 40-year career observing the unique growth patterns of bacteria: "Bacteria are small, not stupid" (Shapiro 2007).

1.2 From Psychoneuroimmunology to Microbial Endocrinology

1.2.1 Theoretical Reflections

During the late 1980s to the early 1990s, I, as well as many others, were involved in the examination of the ability of stress to affect immune responsiveness (Peterson et al. 1991). The field of Psychoneuroimmunology (PNI), founded by Robert Ader and Nicholas Cohen in 1975 (Ader et al. 1995; Ader and Cohen 1975), was just emerging from its infancy into mainstream thinking. That most ambiguous of biological terms, stress, was taking center stage not only in scientific thought but also in the public's perception of immediate, potentially controllable, factors that determined health and well-being. Both in the scientific and public spheres, stress has for many decades been negatively associated with health in general. The demonstration that psychological stress could impact the generation of an immune response (Ader et al. 1995), coupled with reports which showed neural innervation of immune organs such as the spleen (Felten et al. 1990), led to the realization that two seemingly disparate disciplines, one immune and one neural, interacted with each other and that interaction was critical in homeostasis and disease. While the need for such interdisciplinary research is well-recognized today as intrinsic to the study of health (witness the priority of interdisciplinary funding initiatives from the National Institutes of Health), the obviousness of such an approach was less evident in the 1970s to the early 1990s. I can well recall at conferences heated discussions from leaders in the immunology field arguing against the inclusion of neural or endocrinological factors (and certainly not something like psychological which was considered not as scientifically rigorous as a "hard" science) in the study of immune responsiveness. The advent, and increasingly accessibility, of molecular biological tools was beginning to make inroads into deciphering the mechanisms governing the generation of an immune response. Whereas immunological pathways had in the past been deciphered through the study of cell to cell interactions, molecular biological tools afforded a new way to examine such pathways and cellular

immunology began to yield to molecular immunology (with the attendant changes in departmental names). The argument by many of these immunological leaders was that with these new tools we were just beginning to understand the complexity of the immune system and to add onto that the complexity of the neural and endocrinological systems, let alone the even more unknowable psychological factors, would be scientifically "unwise" (actually, more descriptive terms were used at the time) and impede progress. Once we understood the mechanisms governing the immune system, as was the mainstream consensus at the time, only then should we tackle any interactions between different disciplines.

The recounting of the beginnings of PNI is relative to the origins of microbial endocrinology for a number of reasons. First, and foremost, the realization that an interdisciplinary approach was needed if a fuller understanding of the mechanisms that govern immune responsiveness in the host was to be achieved. That no one biological system operates in isolation of another may, on the face of it, be self-evident today; such was not the case even a quarter of a century ago. Since immunological phenomena, such as the production of antibodies, could occur in a completely *in vitro* setting (e.g. Mishell–Dutton culture) where no brain or endocrinological organs are present, why should the products of such systems, i.e. neuroendocrine hormones, be needed for an immune response? Thus, the predominant reasoning was that the immune system was a free-standing biological system that could operate in the absence of any other system. The recognition of neuroimmune interactions as being critical to the development and maintenance of immune responsiveness in an individual can best be seen in the emergence of PNI and the associated neuroimmunology-related field over the past two decades (Irwin 2008).

In many ways, microbial endocrinology has gone, and continues to go through, similar growing pains as that experienced by PNI. Cannot bacteria grow and be studied *in vitro* in the absence of any nervous or endocrinological components? Is such a question no different contextually from that which immunologists once asked of the relevance of neurohormones to the study of immunology? One of the "dirty little secrets" of the time in immunology was that the ability to demonstrate *in vitro* immunological phenomena, such as the generation of antibodies in a Mishell–Dutton system, and hence the independence of immunology from other biological systems, was that multiple lots of a key media component needed to be first screened to find the one "magical" lot that worked best. Once that lot was identified, multi-liter shipments would be ordered and stored for future use. That key media ingredient was fetal bovine serum, itself a rich compendium of neuroendocrine hormones. The realization that endocrine components were necessary to even immunological phenomena, such as antibody formation, underscored the need to study the role of such neuroendocrine influences in the individual.

That the implications of such a connection between media components and sustainability of a biological reaction was not fully recognized at the time is immediately applicable to microbiology and is best illustrated by the response engendered the first time the microbial endocrinology concept was presented at a scientific meeting. At the 1992 American Society of Microbiology 92nd General Meeting in New Orleans, I gave a 10 min slide presentation entitled "Modulation of gram-negative

bacterial growth by catecholamines" (Lyte and Freestone 2009; Mullard 2009). By the time I presented as last speaker in the session, there were only two people in the audience and the two session chairs, one of which was a well-known chair of a large microbiology department. After speaking for about 2 min about the presence of neuroendocrine hormones in bacteria and the need for an interdisciplinary approach to understanding the pathogenesis of infectious disease, one of the audience members left leaving only a solitary person in a room meant for a few hundred people. That audience member happened to be my laboratory technician, Sharon Ernst, who was a co-author on my second microbial endocrinology-related paper. At the finish of my talk, one of the chairs (not knowing I was lecturing to my own technician), evidently felt duty bound due to the presence of an audience member to ask a question, which (to paraphrase) was "why would anyone want to grow bacteria in a serum containing medium containing hormones when such good rich media exist such as tryptic soy broth and brain heart infusion". My answer (again paraphrasing from memory) was simple and still encapsulates one of the underlying tenets that have driven the creation of microbial endocrinology: "… because we do not have tryptic soy broth and brain heart infusion media floating through our veins and arteries and until we use media that reflects the same environment that bacteria must survive in, then we will never fully understand the mechanisms underlying the ability of infectious agents to cause disease".

1.2.2 Experimental Observations Leading to Microbial Endocrinology

The involvement of PNI in the creation of microbial endocrinology went far beyond the theoretical aspects described above. By 1992 I had obtained my first NIH grant which embodied a PNI approach examining the mechanisms by which stress could affect susceptibility to infectious disease. Although stress had been well recognized to affect susceptibility to infections for nearly 100 years (Peterson et al. 1991), I sought to identify relevant immune-based mechanisms through the use of the ethologically-relevant stress of social conflict (Fig. 1.1), instead of the more artificial stressors such as restraint stress or electric shock, which did not reflect any sort of stress that an animal would have any evolutionary experience (Miczek et al. 2001). Among my early findings was that social conflict stress induced an *increase* in those immune functions, notably phagocytosis, that are a first-line against infection (Lyte et al. 1990b). From an evolutionary perspective, the finding of increased immune responsiveness against infection made perfect sense. If an animal is wounded, then bacterial infection would almost certainly be encountered. It made little sense from the animal's perspective to have immune responsiveness decreased at a time that it was presented with an infectious challenge to its survival. What would be needed during this time of acute stress would be heightened immune activity which was what the social conflict study had shown.

Fig. 1.1 Social conflict in mice is conducted by the simple placement of a group-housed mouse also known as an "intruder" (in picture, *black*) into the cage of a singly-housed mouse, also known as the "resident" (in picture, *white*). The resident will engage the intruder ultimately resulting in the "defeat" of the intruder as shown by the limp forepaws and angled ears. Once the intruder assumes the defeat posture, the resident then disengages and at this point the intruder is removed. The social conflict procedure is done under reversed day-night light cycle using low level red light for illumination. For a fuller description of social conflict procedure see Lyte et al. (1990b) and Miczek et al. (2001). The social conflict procedure is done under reversed day-night light cycle using low level red light

However, this surprising result presented a paradox. If immune responsiveness is increased during time of acute, ethologically-relevant stress, then why is the animal more susceptible to an infectious challenge? Most of the literature over the last century had indeed shown that stressed animals did exhibit increased susceptibility to infectious disease challenge (Peterson et al. 1991). With that in mind, I conducted a series of experiments in 1991–1992 in which social conflict stressed animals were challenged with oral pathogens such as *Yersinia enterocolitica*. The results of those experiments showed the surprising result of increased mortality in stressed animals as compared to home cage controls (Fig. 1.2). Shouldn't these animals which showed greater than a 500 % increase in phagocytic capacity (Lyte et al. 1990a, b) also display increased resistance to infectious challenge and not the increased mortality (Fig. 1.2)?

It was these sets of experiments that in 1991–1992 led me to re-consider the whole concept of stress and susceptibility to infectious disease not from the perspective of the animal, but from that of the infecting bacterium. For a number of reasons, the infecting organism is as highly stressed, if not more so, than the stressed host. First, most infectious agents, such as food-borne pathogens have survived food preservation and cooking steps that result in a damaged cellular state. Upon entrance into

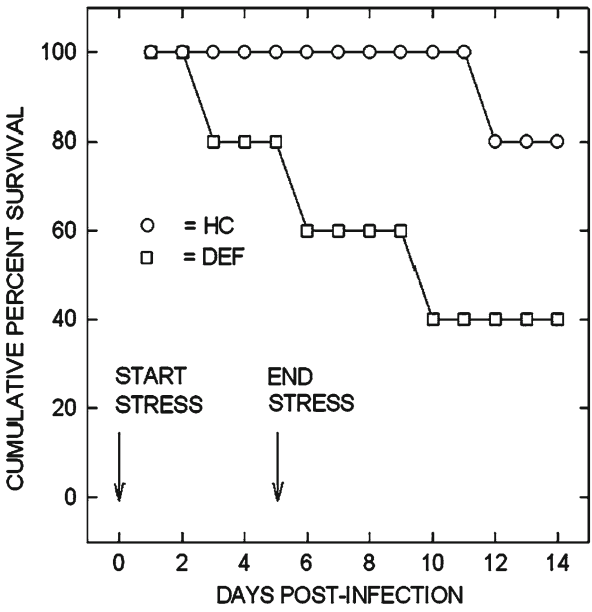

Fig. 1.2 Animals were per orally challenged with *Y. enterocolitica* immediately prior to social conflict stress (DEF, defeated, *squares*) or only handling and transport into procedure room (HC, home cage controls, *circles*). The stress or handling was conducted once per day for 5 days and percent survival followed for 14 days

the host, the infecting bacterium must survive the host's physical defenses such as stomach acid and then survive and proliferate within the gastrointestinal tract amid the trillions of indigenous bacteria which rigorously maintain ecological balance among various species through means including, for example, the elaboration of bacteriocins (Riley and Wertz 2002). Central among the factors that influence the ability of any infecting microbe to survive in a host is the capacity to recognize its environment and then employ that information to initiate pathogenic processes (i.e. adherence onto epithelium) and proliferate. The central question then became, what host-derived signals would be available to an infecting bacterium that could be used to the bacterium's own advantage and ultimately survival within the host? It was at this point that I made the decision to eliminate (for the time being) the role of immunology in addressing the effect of stress on the pathogenesis of infectious disease and instead to concentrate on the role of stress on the infecting bacterium within the hostile environment of the host. In other words, were there direct effects of the *host's* stress response on the bacterium?

Critical to the above line of reasoning was an overlooked phenomena of infectious disease as experienced in nature (real-world) as opposed to the laboratory. That aspect specifically concerns the dose of infectious organisms that are needed to effect overt disease in the host. It is well established in food microbiology that the number of infecting organisms needed to cause food-related gastrointestinal infection can be as low as 10 bacteria per gram of food (Willshaw et al. 1994). However, in the laboratory, the challenge of animals with infectious bacteria can well go as high as $10^{10–11}$ bacteria or colony forming units (CFU) per mL. Further adding to

this discrepancy between real-world and laboratory infectious doses, is that on average a mouse weighs 20–25 g while, on average a human weights 70 kg individual, meaning that the dosage a laboratory animal receives is many-fold greater than what is experienced by an individual. Over the last century a number of investigators have raised the issue of whether non-ecologically relevant doses of infectious organisms can provide complete understanding of the mechanisms that underlie the pathogenesis of infectious disease *in vivo* (Smith 1996). In a similar fashion, this same question can also be raised regarding *in vitro* studies which utilize high ($>10^4$ CFU per mL) bacterial inoculums. Not unlike the question of how a *single* individual may respond to a new environment as compared to how a large *group* of individuals may respond to the same new environment, the survival behavior of low numbers of bacteria within the new environment of the gastrointestinal tract may radically differ from that of large numbers of bacteria. This social aspect of bacterial behavior represents the newly emerging field of sociomicrobiology (Parsek and Greenberg 2005; West et al. 2006). Specifically, the environmental signals that *single* or low numbers of bacteria may look for markedly differ from that sought by high numbers of bacteria. And in addition to the above point of low, not high, numbers of bacteria which contaminate food, this also applies to the vast majority of infections in general in which infecting doses of bacteria are small ($<10^4$ CFU) in number.

Thus, from the outset one of the guiding principles in microbial endocrinology has been the use of low bacterial numbers (1–10^3 CFU per mL) coupled with a medium that is reflective of the *in vivo* milieu. Other guiding principles, such as the combination of neuroendocrine hormones and bacteria under study should be matched such that each is found to occur in the same anatomical region *in vivo*, have also been formulated. In addition to the chapters contained in the present book, the reader is further directed to a comprehensive review which thoroughly discusses the methodological aspects of conducting microbial endocrinology-related experiments (Freestone and Lyte 2008).

The choice of the initial neuroendocrine hormones for the first experiment was based on the stress response itself and the well-known increase in catecholamines (Woolf et al. 1992; Gruchow 1979). Further, the stress-induced release of catecholamines had been one of the primary mechanisms that had been proposed in PNI-related research to account for the ability of stress to suppress immune responsiveness and hence increase susceptibility to an infectious challenge (Ader and Cohen 1993; Webster Marketon and Glaser 2008). As has been recognized for many decades, the induction and sustained release of the catecholamines, especially norepinephrine, occurs during many forms of stress extending from psychological to surgical (Fink 2000). The Gram-negative bacterium, *Y. enterocolitica* was chosen as the first bacterium to test whether a neuroendocrine hormone, namely norepinephrine, could have *direct* effects on growth. The results of this initial experiment in 1992, which was carried out in liquid culture using small 60 mm Petri dishes, combined a low inoculum of *Y. enterocolitica* (33 CFU per mL of serum-supplemented SAPI) with norepinephrine, epinephrine or diluent (Fig. 1.3). In many ways this experiment, which was the proverbial "shot in the dark", is the one that has led through the many years to the creation of this current book. As shown in Fig. 1.3, there is a very small

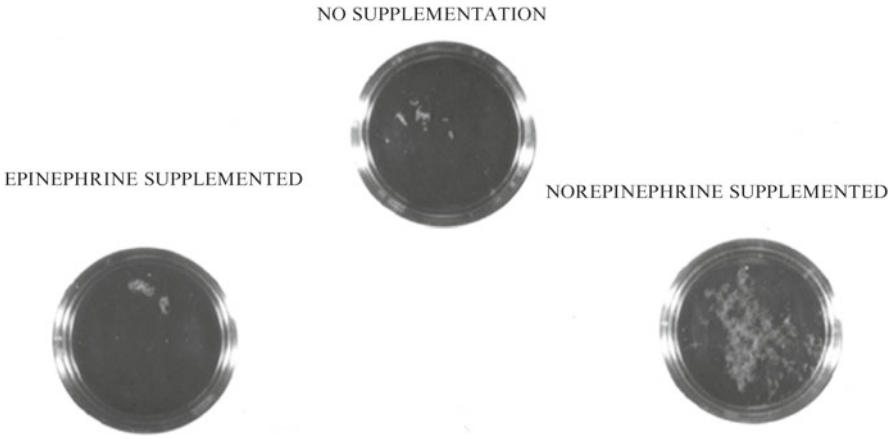

Fig. 1.3 The experiment that launched the field of microbial endocrinology. *Yersinia enterocolitica* culture plates in 1991 showing that bacterial growth in serum-based medium was enhanced in the presence of the neuroendocrine stress hormone norepinephrine, but not epinephrine or control diluent

amount of visual growth evident in both the control and epinephrine supplemented plates (indicated by arrows). However, in the norepinephrine supplemented culture, there is dense growth throughout. To this day I still remember my excitement at seeing these results. And from that day on, I effectively ceased looking at PNI-related phenomena and instead turned my research direction to the study of neuroendocrine-bacterial interactions and the creation of the field of microbial endocrinology.

1.2.3 Gaining Acceptance of Microbial Endocrinology

As can often be the case in any endeavor which seeks to introduce a paradigm-shift in thinking, the introduction of neuroendocrine-bacterial interactions as a hitherto unrecognized mechanism in the pathogenesis of infectious disease was met not only with initial skepticism, but also downright hostility. At a mid-1990s meeting in Toronto that focused on the role of neuroendocrine mediators and immunity in drug addiction, I gave a microbial endocrinology-based lecture as part of a session on stress and its relationship to drug addiction sequelae such as increased prevalence of infectious disease in drug addicts. At the conclusion of my talk before I could take any questions, the session chair addressed the audience and said that my ideas were so radical that they should not be taken seriously and the audience should in essence forget what I just presented. More than one member of that audience has approached me over the years to recount that episode and the shock of the audience being told to disregard what they had just heard as well to ask why I didn't get mad (which I didn't). Such opposition, although admittedly more restrained, was also

encountered during the early years in terms of gaining acceptance into the scientific literature. I have been told by more than one individual that the integration of microbial endocrinology into mainstream infectious disease research would have been accelerated if I had chosen to publish in more microbiology-oriented journals. However, my choice to publish in journals not typically read by microbiologists was dictated not by choice, but instead by necessity. My early attempts to publish in basic microbiology-based journals were universally met with rejection. Undoubtedly, while one may take the convenient rode of blaming the reviewer for failure to consider a highly interdisciplinary approach where it is often not possible to address all the questions regarding each of the fields, I shall instead take a fair share of the blame since it is also the responsibility of the author to educate the reader of the need to go beyond traditional thinking.

With that said, I have also come to recognize that one of the defining reasons that these early papers were rejected from basic microbiology-centric journals was the reliance on phenomenology rather than mechanisms. My own training in the clinical laboratory sciences and subsequent work in hospital laboratories before entering graduate school in 1977, ingrained in me a powerful sense of the clinical side of microbiology. And that side is one that is grounded in growth for without evident growth and sufficient numbers of bacteria little can be done, even today, to diagnosis suspected bacterial disease. Thus, it seemed to me at the time (and still does today) that the ability to show growth-related effects of neuroendocrine hormones on bacteria would have profound implications for the study of the host factors which influence susceptibility to infectious disease. However, I was surprised that this was generally not the case. A similar refrain ran through those early reviews that the demonstration of effects on growth were phenomenological in nature and what needed to be shown was the mechanism(s) by which neuroendocrine hormones could influence bacterial physiology. Due largely to the availability of an ever growing arsenal of molecular biological techniques, phenomenology was to be eschewed in favor of dissecting molecular mechanisms. While I do not mean to begrudge nor demean the value of mechanistic studies, one may argue that many of the advances in the treatment of disease have been made through the observation of phenomena for which no mechanism at the time of discovery was available. Antibiotic development owes itself largely to the observation of phenomena. While the requirement for molecular analyses currently reigns dominant in the majority of first-tier microbiology journals, the relegation of phenomenological studies to the status of second-class research ignores its historically pivotal role in fueling scientific and medical advances. A number of articles examining the failure of genomic-based strategies to lead to the discovery of new antimicrobials that ultimately make the transition from the lab bench to clinic have addressed this very point (Finch 2007; Barrett 2005).

My reasoning for discussing the relative merits of phenomenology versus molecular analyses is not to point out my own shortcomings in the area of molecular analysis, but instead to offer a cautionary note to other researchers who may choose to explore microbial endocrinology. Catecholamines, which to date have been the principal neuroendocrine hormones that have been examined in the microbial

endocrinology field by virtue of their prominence in the stress response, represent but a tiny sliver of the spectrum of neuroendocrine hormones that can be examined for potential interaction with both pathogenic as well as commensal bacteria. For example, gamma amino butyric acid (GABA), the primary inhibitory neurotransmitter in the mammalian brain, is produced in such large amounts by bacteria in the gut that a role for bacterial-derived GABA has been proposed to account for the altered brain function (encephalopathy) that is part of the pathogenesis of advanced liver disease and sepsis (Minuk 1986; Winder et al. 1988). In fact, GABA produced by bacteria, such as those contaminating a distilled water apparatus, have been found not only to confound neurotransmitter binding studies with mammalian cells (Balcar 1990), but also to possess a high affinity binding protein that resulted in one of the first bioassays for GABA that was entirely bacterial-based (Guthrie and Nicholson-Guthrie 1989; Guthrie et al. 2000). In this book, Chap. 4 by Victoria Roshchina provides an exhaustive review of the wide breadth of neurohormones that are found in prokaryotes that we otherwise only associate with multi-cellular eukaryotic systems.

By utilizing a microbial endocrinology approach, researchers can further our understanding of how host and bacteria, both commensal and pathogenic, interact in the gut (or at other sites). That approach, in turn, could provide insights into not only homeostasis but also other medical conditions that involve gut pathology that upon verification could enable the design of new innovative medical interventions. Although researchers realized more than 100 years ago that the mammalian gut is innervated, how this system interacts with the gut microbial flora remains largely a mystery. Further, large amounts of neurochemicals are produced within the gut that find its way into the gut lumen where the possibility of interactions with the gut microflora exist and remain largely unexplored. For example, large quantities of serotonin are produced by the gut that can be recovered from the lumen, although the physiological reason for this production are not well understood. Could it be that serotonin produced by the mammalian gut has some hitherto unknown interaction with a specific part of microbial population? Thus, examination of any such serotonin-bacterial interaction will depend on both *phenomenology* and molecular analyses to provide as complete a picture as possible of the relevance of microbial endocrinology to both homeostasis and disease.

Further, the bi-directional nature of bacterial-microbial interactions contained with the theory of microbial endocrinology also suggests that bacteria can influence mammalian function. Work utilizing metabolomics to compare the blood metabolic profile of conventional-reared and germ-free mice revealed that the gut microbiome contributed to the concentration of neuroactive components in the circulation (Wikoff et al. 2009). That the presence of a microbial community within the gut, and inherent interactions between the host and gut microflora, is crucial to an animal's neurological health was demonstrated in 2004 when Nobuyuki Sudo and colleagues at Kyushu University in Japan examined the role of microbial colonization on the hypothalamic-pituitary-adrenal response to stress in gnotobiotic, germ-free and conventionally-reared mice (Sudo et al. 2004). Not only did the development of host neural systems that control the physiological response to stress depend on

postnatal microbial colonization of the gut, but reconstitution of gnotobiotic mice with feces from specific pathogen-free mice altered their subsequent neurohormonal stress response. Additionally, Li et al. demonstrated that diet-induced alteration of gut microbial diversity can even affect memory and learning in mice (Li et al. 2009). Thus we are just beginning to understand the degree to which microbial diversity is crucial to the development and regulation of normal gastrointestinal function. Does gut neuronal activity influence local bacterial ecology and vice versa?

1.3 Collaboration and Dissemination

The development of any field is often dependent on the interactions and potential collaborations with others. Since the initial first sole authored reports in which the concept of *direct* microbial endocrinology-based interactions (Lyte 1992, 1993) was reported and the theory of its proposed role in health and disease was discussed, it has been the subsequent efforts of graduate students, technicians and fellow scientists that has been instrumental in the growth of microbial endocrinology. While over the course of time some of these collaborations have remained strong, for example, graduate students some of whom later became collaborators in the examination of microbial endocrinology in the microbiota-gut-brain axis (Galley et al. 2014; Lyte et al. 1998), others ended often after running their natural course while others came to a more acrimonious ending. As this chapter is titled, in part, "a personal journey", the realization that one highly productive collaboration had abruptly ended occurred one late afternoon after a literature search just by happenstance referenced a document in a European data that upon reading, and seeing mutual experimental ideas discussed but no mention that it was indeed collaborative, felt like stepping off a curb, turning to one's side, and getting hit by a high speed truck. The pursuit of science is not often the straight and collegiate course one imagines as a student.

A critical point in the development of microbial endocrinology turned out to be a fortuitous meeting at the 1995 First International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases held in Rapid City, South Dakota. Following my presentation, I was approached by a bearded and pony-tailed Richard Haigh, at the time a Ph.D. student at Leicester University in the United Kingdom. Richard's interest in my work served as the bridge to Primrose Freestone who was a post-doctoral fellow in his lab in the then Department of Microbiology and Immunology within the medical school. During this time I also consider myself fortunate to have helped interest other investigators to examine bacterial-neuroendocrine interactions. For example, during microbiology meetings both in the United States and in Japan at which I and my colleagues had presentations, I was approached by James Kaper of the University of Maryland and his then postdoctoral fellows Jorge Girón and Vanessa Sperandio. Following stimulating conversations with them regarding my concept of bacteria recognizing hormones and the potential

of microbial endocrinology, as well as instructions on how to design experiments utilizing neurochemicals, I sent shipments from my lab of neurochemicals to help with initial experiments. Nearly 2 years from the time of my first discussion with James Kaper, Vanessa Sperandio and her colleagues published their landmark paper in PNAS demonstrating the ability of catecholamines to influence quorum sensing in *Escherichia coli* O157:H7 (Sperandio et al. 2003).

It is axiomatic that the development of any newly emerging field must of necessity rely on the willingness of researchers to freely exchange ideas. Often such exchange is not rewarded with any acknowledgement nor at times proper citation of papers that were instrumental in the founding of the field. As seems to be increasingly the case in science in general, this may be an unfortunate sign of the current times. Regardless, the further theoretical and experimental development of any field must continue to rely on the free exchange of ideas even though the practical aspects of such scientific exchange often do not match the hoped for ideal embodiment of such ideals, especially the regard for proper citation.

Probably one of the greatest fears when one believes to have discovered a new discipline is whether one is really the first. Nearly 5 years following the 1992 publication of my initial papers that helped lay the foundation for microbial endocrinology (Lyte 1992; Lyte and Ernst 1992) I became aware of a reference by the noted physician and essayist Lewis Thomas in his book "The Lives of a Cell: Notes of a Biology Watcher" in which he wrote that one of the forgotten secrets of microbiologists was that the only way to get *Clostridium perfringens* to establish in mice, in order to evaluate potential antimicrobial agents in the treatment of gas gangrene, was to co-inject epinephrine with the *C. perfringens* (Thomas 1974). This passing revelation, written by Thomas to illustrate the complexity of biological systems in general, hit me like a thunderbolt that my fear of not properly citing those before me had been realized. What followed turned out to be a journey into a rich history documenting the ability of neuroendocrine hormones to influence the pathogenesis of infectious disease and the many attempts that have been made to understand the phenomena (the majority of these studies have been referenced in my 2004 review, Lyte 2004). In retrospect, given the usage of neuroendocrine hormones such as the catecholamines dating as far back as 1930 in the treatment of clinical conditions ranging from urticaria (itching) (Renaud and Miget 1930) to their current prominent role in the maintenance of cardiac and kidney function in critically-ill patients (Singer 2007), it should not come as that much of a surprise that there has been ample opportunity for the observation of neuroendocrine-bacterial interactions in infectious disease. Indeed, a report detailing the ability of catecholamines to increase aerotolerance of *Campylobacter jejuni* is instructive of often seminal reports whose significance is not appreciated until many years later (Bowdre et al. 1976; Hoffman et al. 1979). The study of this past literature is highly instructive not simply as only an historical narrative, but as importantly as a guide to future research design from a translational medicine viewpoint since it provides evidence of the role of microbial endocrinology both in health and disease in both animals and humans.

1.4 Microbial Endocrinology and the Microbiota-Gut-Brain Axis

In the first edition of this book, I had offered some future predictions where the field might be heading as well as some cautionary notes (Lyte 2010a). In the intervening 5 years there has been continued growth of the field in the realm of infectious disease as can be well seen in the new and updated chapters in this second edition. At the same time, an increasing number of reports began to appear in the literature suggesting the recognition that the gut microbiota could influence aspects of host behavior (Bercik et al. 2009, 2010; Collins et al. 2013; Neufeld et al. 2011a; Bravo et al. 2011; Cryan and Dinan 2012; Desbonnet et al. 2013; Lyte 2011, 2013b; Bailey et al. 2011). This ability of the microbiota to influence brain function via what has been termed the microbiota-gut-brain axis (Lyte and Cryan 2014), is covered in-depth by the addition of a new chapter to this second edition devoted exclusively to this axis (Chap. 15). The inclusion of this chapter reflects the rapid growth of research into the microbiota-gut-brain axis that was still only nascent at the time of the first edition in 2010 (for comprehensive review see chapters in Lyte and Cryan 2014).

Notwithstanding the recent rapid growth and recognition by many (but certainly not all) in the wider scientific community, as in the case of the initial reports dating back to 1930 of bacteria responding to catecholamines, the proposal that gut microbiota could directly influence brain function has a long history (for comprehensive reviews see Bested et al. 2013a, b, c). The "health" of the colon was seen as paramount and given the bacterial load, as well as the ability at the turn of nineteenth century to culture exclusively aerobic bacteria, it was the obvious anatomical region to link to the brain. Any disturbance in the composition of the colonic contents was linked to alterations in psychological well-being (Bested et al. 2013a). Individuals such as the surgeon H.R. Kellogg (who would later found a cereal company bearing his name to further the idea of colonic health) advocated the removal of the colon itself and its perceived harmful contents, as a way to improve mental health: "*Should the colon be sacrificed or reformed … I have labored constantly and earnestly to devise and perfect methods for changing the intestinal flora …*" (Kellogg 1917). The inherent difficulty in attempting to manage the composition of the gut microbiota was well-recognized over 100 years ago as it is today: "*The control of man's diet is readily accomplished, but mastery over his intestinal bacterial flora is not … They are the cases that present … malaise, total lack of ambition so that every effort in life is a burden, mental depression often bordering upon melancholia … A battle royal must be fought and when this first great struggle ends in victory for the Bacillus bulgaricus it must be kept on the field of battle forever at guard …*" (Stow 1914).

The modern concept of a microbiota-gut-brain axis has relied on an ever-growing number of studies which have demonstrated the ability of bacteria to influence brain function (Bercik et al. 2009, 2010; Collins et al. 2013; Neufeld et al. 2011a; Bravo et al. 2011; Cryan and Dinan 2012; Desbonnet et al. 2013; Lyte 2011, 2013b;

Bailey et al. 2011). Initial studies utilized the introduction of novel bacterial species into the gut microbiota that did not cause infection nor activation of the immune system at the doses administered per orally (Lyte et al. 1998). In these studies, the introduction of novel bacterial species such as *Campylobacter jejuni* into the gut microbiota of mice resulted in activation of specific neural centers in the brain (Gaykema et al. 2004) and that a consequence of this was the induction of anxiety-like behavior (Lyte et al. 1998). The involvement of the vagus nerve, the largest nerve in the body which has numerous branches innervating the gut, in carrying signals from the gut to the brain was demonstrated when partial sub-diaphragmatic vagotomy abrogated the ability of novel bacteria to induce a behavioral response (Goehler et al. 2005). Critical work performed by others, notably the transfer of behavioral phenotype by adoptive transfer of the microbiota has been instrumental in identifying the role of the microbiota-gut-brain axis in behavior. In these experiments performed, the microbiota from a mouse strain displaying an anxious phenotype was adoptively transferred via fecal microbial transplant into another strain which typically exhibited normal, non-anxious, behavior (Collins et al. 2013). The mice which previously displayed only normal behavior, evidenced anxiety-like behavior that was identical to that of the anxious mouse strain from which the fecal microbial transplant was obtained (Collins et al. 2013). The use of germ-free versus conventionally-housed mice has also figured prominently in studies which have examined the ability of the microbiota to influence brain function (Neufeld et al. 2011b; Diaz Heijtz et al. 2011). Indeed the first study to identify the ability of microbiota from specific-pathogen free mice to be adoptively transferred into germ-free mice was that performed by Sudo et al. who showed that following adoptive transfer the hypothalamic-pituitary response to mild restraint stress was attenuated as compared to non-colonized control germ-free animals (Sudo et al. 2004). While such studies involving germ-free animals have been instrumental in furthering the concept of the microbiota-gut-brain axis, a note of caution regarding their use in microbiome-related studies has been raised (Hanage 2014).

Given the capacity of bacteria to produce a wide spectrum of neurochemicals with known ability to affect brain function and behavior, it is therefore reasonable to propose that the *in vivo* production of neurochemicals by bacteria could influence brain function, and hence behavior, by either of two possible routes. The first would be through the production of neurochemicals that could act locally within the gut by interacting with cognate receptors belonging to the host enteric nervous system. Such interaction would then be communicated to the brain most likely by the vagus nerve. The second route would also involve the production of neurochemicals known to influence behavior, but this route would involve the uptake of the neurochemicals into the portal circulation and its eventual transport into the brain.

With either route, the first question that must be addressed is whether bacteria in the host can produce neurochemicals of sufficient concentration to influence host neurophysiology. Since the publication of the first edition of this book, that question has been answered by Asano and colleagues who published the landmark paper describing the *in vivo* production of dopamine and norepinephrine by gut bacteria (Asano et al. 2012). In this study, luminal levels of catecholamines in the gastroin-

testinal tract were measured in specific pathogen-free, germ-free, and gnotobiotic mice. Asano et al. reported that while the catecholamines, norepinephrine and dopamine, were produced in appreciable physiological amounts in the luminal contents of specific pathogen free mice, in germ-free animals substantially lower amounts were detected (Asano et al. 2012). Critically, whereas the majority of catecholamines in pathogen-free animals were structurally determined to be free and biologically active, those found in germ-free animals were present in a biologically inactive, conjugated form. Inoculation of germ-free animals with the flora from specific pathogen free mice resulted in the production of free, biologically active, catecholamines within the gut lumen. As such, this report clearly established that *in vivo* the microbiota is capable of producing neuroendocrine hormones that are commonly only associated with host production. That these substances also are intimately involved in host neurophysiology provides solid evidence that the fields of microbiology and neurophysiology do intersect with attendant consequences for both host and microbiota as further discussed below regarding the ability of the gut microbiota to influence brain function.

Subsequently, the in-depth examination of a large number of bioactive metabolites in the ceca of germ-free and specific pathogen-free mice, has revealed the presence of a large number of metabolites that function in the mammalian system as neurochemicals in cell-to-cell signaling within the nervous system (Sridharan et al. 2014). By utilizing probabilistic pathway construction, Sridharan and colleagues were able to identify both predicted and detected metabolites whether their origin was host-specific, microbiota-specific or expressed by both. Among those compounds that they identified as potentially having the capacity to influence gut-brain communication was serotonin and its precursor 5-hydroxy-L-tryptophan. Intriguingly, levels of each differed between germ-free and specific-pathogen free mice (Sridharan et al. 2014). The microbiota-derived presence of serotonin is not surprising give the previous report of Wikoff et al. which demonstrated that the plasma level of serotonin was nearly threefold higher in conventionally-microbiota colonized mice than in germ-free animals (Wikoff et al. 2009). Despite this reported finding, Wikoff et al. did not want to describe this as being due to production of serotonin by the gut bacteria "… because the production of serotonin by characterized enteric bacterial species has not been described (Wikoff et al. 2009)". In fact, the report by Sridharan et al. (2014) of gut microbiota production of serotonin is in fact not the first report. Production of serotonin had been noted in the intestinal tract of the parasitic nematode *Ascaris suum* nearly three decades prior (Hsu et al. 1986; Shahkolahi and Donahue 1993).

Given, as demonstrated by the above reports that bacteria can produce neurochemicals of sufficient quantity to influence host physiology, the next question that must be raised concerning whether that production occurs in an neuroanatomical region of the gut where interaction with cognate receptors belonging to the ENS can occur. It would only be through such local receptor-based binding of cognate neurochemical receptors on either host neural elements belonging to the ENS or receptors on other host enteric cell types (i.e., enterocytes) that signaling to the brain predominantly through the vagus could occur. To date, there have been no published

studies which have examined the composition of microbial communities in relation to neuroanatomical regions in the gut.

Likewise, there are still no publications which have examined whether neurochemicals produced in the host gut by luminal bacteria can be of sufficient quantity that following uptake through the gut into the portal circulation making their eventual way to the brain where they could influence function. There are a number of barriers that may preclude neurochemicals produced by gut bacteria in the gut lumen from ever being able to reach the brain. First and foremost is that the brain parenchyma actively excludes a number of neurochemicals that are known to be produced by bacteria, such as dopamine and GABA, from crossing the blood brain barrier. However, neurochemicals produced by gut bacteria may not have to completely transit through the bloodstream to the brain to ultimately affect brain function since they may interact in the periphery with sensory fibers present in organs that directly communicate with the central nervous system (Cryan and Dinan 2012).

In considering which of the two routes by which neurochemicals produced by gut microbiota may affect brain function, it seems more probable for the first route wherein microbiota-produced neurochemicals in the gut interact with components of the ENS to influence the brain and ultimately behavior. Future research examining the second route of gut microbiota-derived neurochemicals transiting through the bloodstream to the brain will hopefully provide the data necessary to validate it as a mechanism by which the microbiota-gut-brain pathway may influence host behavior.

1.5 Microbial Endocrinology and Probiotics

The concept of probiotics as neurochemical drug-delivery vehicles has been proposed (Lyte 2011). More recently, Dinan et al. have proposed that probiotics that possess the capacity to deliver therapeutic levels of neurochemicals that could be potentially used to treat psychiatric illness should be termed "psychobiotics" (Dinan et al. 2013). The ability of probiotics to make neurochemicals, not unexpectedly, goes back over many decades into the early part of the twentieth century. One of the earliest descriptions was the production of acetylcholine by *Lactobacillus plantarum* (Stephenson and Rowatt 1947) as well as reports of isolation of acetylcholine-producing strains from the human gastrointestinal tract (Habs 1937–1938).

Probiotics such as those belonging to the genus *Lactobacillus* are potent producers of neurochemicals such as GABA (Li et al. 2008). The microbial endocrinology-based theory has been introduced that probiotics function as pharmacological agents and hence function as drug delivery vehicles due to their ability to synthesize hormones, such as GABA (which can directly influence receptors both immune and neural within the ENS and CNS) (Lyte 2011). Evidence to support this microbial endocrinology-based understanding of the mechanisms by which probiotics may influence host behavior and inflammation can be seen in the studies of Bravo et al. (2011) and Thomas et al. (2012). Bravo et al. reported that the ability of the probiotic *L. rhamnosus* to influence emotional behavior in mice was due to alterations in GABA

receptor expression that were dependent on communication from the gut to brain via the vagus nerve since vagotomy negated the effect of the probiotic. Experiments, which sought to examine the mechanisms by which the probiotic *L. reuteri* influenced inflammation in an *in vitro* model system, reported that the suppressive action of the probiotic was due to the production of a neuroendocrine hormone, specifically histamine, that inhibited the production of inflammatory cytokines such as TNF (Thomas et al. 2012). The microbial endocrinology-based hypothesis that probiotic organisms may function as drug-delivery vehicles equally applies to the large endogenous (non-probiotic) population of Gram-positive bacteria residing in the gut. Since many of these bacterial species possess the biochemical machinery to synthesize a neuroendocrine hormone such as GABA (Komatsuzaki et al. 2008), it is reasonable to suggest that the examination of diets rich in the precursors of these neurochemicals may increase the production of the hormone within the gut which in turn may affect both host physiology (i.e. inflammation within the gut) and behavior.

Interestingly, both live and dead probiotics have been shown to be equally efficacious (Iannitti and Palmieri 2010), and the need for actually administering the probiotic itself has been questioned in a report, which has shown that simple administration of the bacterial culture medium in which the probiotic has been grown is sufficient to achieve the same changes in colonic motility as when the bacteria themselves are administered (Bar et al. 2009). An excellent illustration of the capacity of probiotics to produce copious amounts of neurochemicals that can then be employed to alleviate behavioral issues, and not inconsequently replace current pharmacological drugs, can be seen in a study which examined the ability of *Lactobacillus brevis* strain to enrich black soybean milk with GABA (Ko et al. 2013). This GABA-enriched functional food was then fed to rats subjected to a forced swim behavioral test. Forced swim tests, in which animals are placed in a water-containing glass cylinder and the duration of immobility before the animals begin to swim is measured, is a well-recognized test of depressive-like behavior. In this study, it was shown that GABA-enriched soybean milk significantly reduced the immobility time before rats began to swim and was as effective as the selective serotonin reuptake inhibitor fluoxetine as an antidepressant (Ko et al. 2013). Although as a functional food report, this study is not an *in vivo* demonstration of the ability of a probiotic to alter behavior. It does, however, demonstrate that neurochemicals in the gut (albeit consumed as part of a function food) can ultimately influence the brain and affect behavior (Patterson et al. 2014).

1.6 Whither Microbial Endocrinology

In the previous edition I had written that "predictions concerning future progress, such as the usual self-serving admonitions about the inevitability of discoveries leading to improvement of the human condition, are fraught with peril". That is more true now than when the first edition was published in 2010. As I have alluded to in this chapter and elsewhere, there still is a long road to go from simply

proposing a microbial endocrinology-based mechanism, such as in the use of probiotics that secrete neurochemicals, to actually proving the mechanism *in vivo* (Lyte 2011). Indeed, the issue of correlation and causation is one that has been raised concerning the putative role of the microbiota in many areas of health that it was not previously perceived to be involved in, including that of the brain function and by extension behavior. The over-hyping of the microbiome has drawn justified concern (Hanage 2014). And the same admonition must be applied to the study of microbial endocrinology.

Whatever the direction that microbial endocrinology will take will depend on a myriad of factors. Among the most crucial of factors will be experimental design. Separating out the neurochemicals from what the host produces from that of the microbiota has been, and will continue to be, not an easy task. In this respect, it is therefore gratifying to see the recent publication by Sridharan et al. that describes the elegant approach of probabilistic pathway construction that enables the evaluation of what is hot, microbiota or both (Sridharan et al. 2014). Although this subject has been discussed in detail before (Freestone and Lyte 2008), there are some points that bear repeating. First and foremost, the choice of environmental conditions, especially those that concern *in vitro* culture, namely the media with which is chosen to evaluate potential microbiological response(s) to neuroactive agents, will remain the single most important determining criteria to evaluate potential interactions between microbes and neuroactive compounds. The use of rich media, such as Luria-Bertani and tryptic soy broth, to name just two, is likely a self-defeating way to approach evaluation of neuroendocrine-microbial interactions. If the microorganism has everything it needs for survival and proliferation already present in the rich media, why would there be any need to actively seek those environmental signals, such as neuroendocrine hormones, in order to establish where they are and then initiate processes needed for survival in nutrient-limited environments that are not akin to *in vitro* incubation in rich media? The answer is that it is highly unlikely that one would obtain the same response from a microorganism that is exposed to a neurochemical in a rich medium as compared to a nutrient-limited medium.

This above *in vitro* caveat applies equally to the design of *in vivo* experiments. As discussed in more detail in the first published review on the field of microbial endocrinology (Lyte 2004), the non-peer reviewed study which had reported the dramatic ability of norepinephrine to increase the *in vitro* growth of *Salmonella choleraesuis* could not be replicated an *in vivo* animal model in which the authors had implanted a norepinephrine-containing pellet subcutaneously on the back and weeks later infected the animals via an intraperitoneal route with bacteria (Nietfeld et al. 1999). As argued in the 2004 review (Lyte 2004), the authors of the 1999 study (Nietfeld et al. 1999) made a number of experimental design errors regarding rather standard animal physiology. These included, but were not limited to, assuming that the parenteral administration of a specific hormone will result in a constant defined concentration within any one specific organ or organ system over a period of weeks and that the body over an extended time frame will not seek to adapt to elaboration of the hormone by increasing the production of specific degradative enzymes whose pathways have been well characterized over the past decades. A simple consideration

of the anatomical distance of the peritoneal cavity into which the bacteria where injected from the subcutaneous space on the back of the neck into which the norepinephrine release pellet was inserted, and the body's immediate system-wide response of increased enzymatic degradation of circulating catecholamines, it would be rather problematic to interpret any changes in bacterial growth rates in terms of any endocrine influence. And since the issue of environmental context is pre-eminent in any discussion of microbial endocrinology, would it not have been more physiologically relevant to have evaluated the ability of a stress-related neuroendocrine hormone to affect the growth of *S. choleraesuis* in the gut, which is the natural site of infection with this organism, rather than the peritoneum?

Microbial endocrinology in many ways represents an interdisciplinary discipline that seeks to understand the role of microbes in health and disease that is driven for the most part by an evolutionary approach (see Chap. 2 for a discussion of the ubiquitous distribution of neuroactive compounds in nature). Although much has been made of the use of the term interdisciplinary (and the many variants such as multidisciplinary), the "take home" message may be that in approaching a study of neuroactive compounds in microbiology, microbial endocrinology represents an opportunity for true interaction of microbiologists with individuals from disciplines not readily associated with the study of microbial processes, such as physiologists, endocrinologists, behaviorists, to name a few. Recognition of the interdisciplinary nature of microbial endocrinology among microbiologists and the opportunities it may afford for understanding the role of the microbiota in health and well-being is increasingly being seen as reflected by reviews such as that by Neuman et al. (2015). This increasing interdisciplinary recognition also extends to the study of the ability of the microbiota to possibly influence appetite and food choices as has been proposed by a number of theoretical papers (Lyte 2013a; Norris et al. 2013; Alcock et al. 2014). Given that foods also contain both the precursors for the synthesis of neurochemicals by host and microbiota as well as neurochemicals themselves (Lyte 2013a), it can be proposed that nutrition may also play an important and largely unexplored role in the ability of the microbiota to influence the host (Fig. 1.4).

The recent advent of the microbiota-gut-brain axis illustrates how microbial endocrinology may grow beyond its early roots in infectious disease. Whether microbial endocrinology will ultimately prove to be a viable mechanism by which the microbiota in the gut can influence brain function has yet to be conclusively demonstrated. However, current work in my laboratory as well as others is intensively examining the possibility. Similarly, the selection and use of probiotics based upon their capacity for neurochemical production is also being intensively investigated by a number of laboratories.

And finally, as the oft-used cliché goes "this is not the end of the story, just the beginning". Prior to the publication of the first edition of this book in 2010, I received a call from a colleague from my past days in the PNI field which, as described earlier in this chapter, helped shape my ideas concerning microbial endocrinology. In recalling my talks from nearly 25 years ago at which I presented the first evidence for the role of neurohormones in the ability of stress to affect the course of bacterial infection

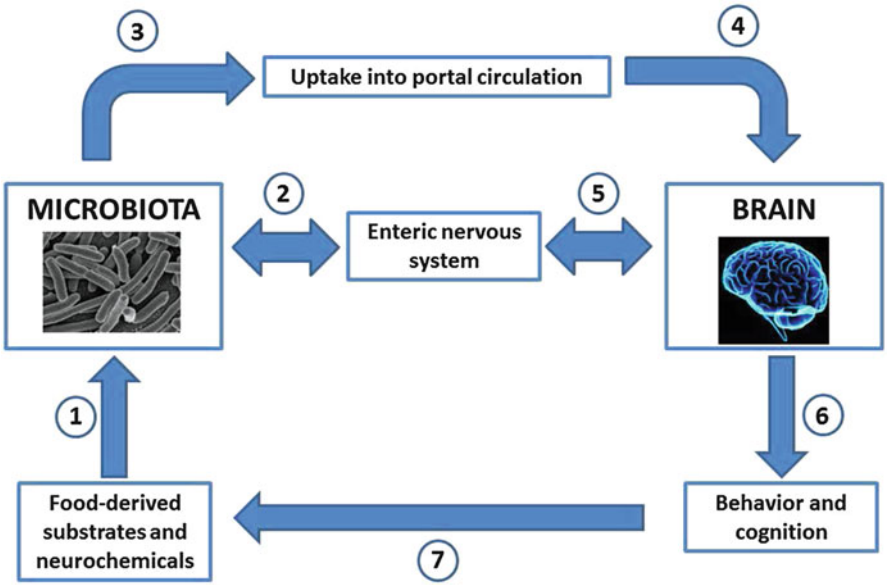

Fig. 1.4 The microbial endocrinology-based pathways by which neuroactive compounds produced by both the host and the microbiota can serve as a mechanism by which the brain and behavior can be modulated within the microbiota-gut-brain axis. Food ingested by the host contains both the substrates needed for neurochemical production by the host and the microbiota as well as fully functional neuroactive components (*1*). The microbiota in the gut is capable of either forming neurochemicals from the substrates present in the ingested food; or responding to the neuroactive food components themselves; or responding to neurochemicals secreted into the gut by components of the host enteric nervous system (*2*). Neurochemicals produced by the microbiota in the gut have two pathways by which to influence the host; they can either be taken up from the gut into the portal circulation (*3*) or they can directly interact with receptors found on components of the enteric nervous system which innervates the complete length of the gastrointestinal tract (*2*). Once in the portal circulation, microbiota-derived neurochemicals can influence components of the nervous system and ultimately the brain (*4*). Microbiota-derived neurochemicals can also influence components of the nervous system such as the brain through enteric nervous system-central nervous system communication (*5*). The result of either pathway (*4*) or (*5*) on the brain may result in an alteration of behavior or cognition (*6*) as well as food preferences and appetite (*7*) (Lyte 2010b, 2013a; Norris et al. 2013; Alcock et al. 2014). This should not be viewed as a one-way direction of only gut-to-brain since the brain may influence the composition of the microbiota through the specific release of neurochemicals into the gut lumen (*2*). Reprinted with permission from (Lyte 2013a)

and the somewhat rocky road that I travelled since that time in terms of recognition by the scientific community, he commented that my days of "wandering in the wilderness" appeared to be over. If that is indeed the case, as I do hope the contents of this book will attest to, then I must add to that sentiment that it has only been with the help of my collaborators, students, and technicians over the years that it has been possible to make that journey.

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Chapter 2
New Trends and Perspectives in the Evolution of Neurotransmitters in Microbial, Plant, and Animal Cells

Victoria V. Roshchina

Abstract The evolutionary perspective on the universal roles of compounds known as neurotransmitters may help in the analysis of relations between all organisms in biocenosis—from microorganisms to plant and animals. This phenomenon, significant for chemosignaling and cellular endocrinology, has been important in human health and the ability to cause disease or immunity, because the "living environment" influences every organism in a biocenosis relationship (microorganism-microorganism, microorganism-plant, microorganism-animal, plant-animal, plant-plant and animal-animal). Non-nervous functions of neurotransmitters (rather "biomediators" on a cellular level) are considered in this review and ample consideration is given to similarities and differences that unite, as well as distinguish, taxonomical kingdoms.

Keywords Acetylcholine • Biomediators • Biocenosis • Catecholamines • Evolution of functions • Histamine • Interactions between organisms • Microorganisms • Non-nervous functions of neurotransmitters • Pharmacology • Plants • Serotonin • Toxicology

2.1 Introduction

Humans cohabitate a "living world" populated with microorganisms, plants, and other animals. The myriad relationships between organisms occurs via what may be here called, "reaction-response" events. The mechanism of reaction-response appears to have a common base in the form of chemical signals—chemicals which

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are identifiable by different cell types and, often too, by organisms of different taxonomical kingdoms. Similar compounds likely to be found in living organisms include acetylcholine, dopamine, norepinephrine, epinephrine, serotonin, and histamine, collectively known as neurotransmitters, and have been found not only in animals (Boron and Boulpaep 2005), but also in plants (Roshchina 1991, 2001a; Murch 2006) and microorganisms (Hsu et al. 1986; Strakhovskaya et al. 1991; Lyte 1992; Oleskin et al. 1998a, b; Tsavkelova et al. 2006; Freestone and Lyte 2008). Thus, the presence of neurotransmitter compounds has been shown in organisms lacking a nervous system and even in unicellular organisms (Roshchina 1991, 2001a, 2010). Today, we have increasing evidence that neurotransmitters, which participate in synaptic neurotransmission, are multifunctional substances that participate in developmental processes of microorganisms, plants, and animals. Moreover, their universal roles as signal and regulatory compounds are supported by studies that examined their role in and across biological kingdoms (Roshchina 1991, 2001a, 2010; Baluska et al. 2005, 2006a, b; Brenner et al. 2006; Ramakrishna et al. 2011). Any organism may release neurotransmitters, and due to these secretions the "living environment" influences every other inhabitant of biocenosis, determining relationships between organisms such as microorganism–microorganism, microorganism–plant, microorganism–animal, plant–animal, plant–plant, and animal–animal. The universal character of neurotransmitters, as well as their occurrence and similarity of functions at the cellular level, should compel the scientific community to rename "neurotransmitters" to "biomediators" in order to better allow the term's application to any living cell—not just those organisms that possess nervous systems (Roshchina 1991, 2001a, 2010). The biomediator concept permits us to imagine the complete evolutionary picture, wherein the neurotransmitter substances are participators in many different cellular processes, including non-synaptic systems of microorganisms and plants. Non-nervous system functions of the neurotransmitters (rather "biomediators" at the cellular level) are analyzed in this chapter, and their respective roles in the different evolutionary kingdoms are compared. This information, gathered from species ranging from microorganisms to plants and animals, may provide insight into key problems in cellular endocrinology, and thereby have implications for understanding both health and disease causation. Such analysis can also undoubtedly provide useful perspectives to help guide the future development of the field of Microbial Endocrinology.

2.2 Occurrence of Neurotransmitters in Living Organisms

2.2.1 Discoveries

Historical chronologies of the neurotransmitters' discoveries are represented in Table 2.1. The first neurotransmitters were the catecholamines found by the American scientist John Jacob Abel at the end of the nineteenth century in extracts from animal adrenal glands. During the years 1906–1914, the existence of neurotransmitter compounds was identified not only in animals, but also in fungal

Table 2.1 Discovery of neurotransmitters

Neurotransmitter	In microorganisms	In plants	In animals
Acetylcholine	Identified independently by Ewins (1914) and Dale (1914) in preparations of ergot spur fungus *Claviceps purpurea* in Great Britain and in bacteria. *Pseudomonas fluorescens* (Chet et al. 1973)	In 1947 Emmelin and Feldberg found this substance in stinging trichomes and leaves of common nettle by biological method, based on muscle contraction	In 1921–1926 the presence of acetylcholine has been established in animals by Loewi and Navratil. But earlier, in 1906 student Reid Hunt (worked in USA laboratory of John J. Abel) discovered it in adrenal extracts of animals
Dopamine	Found in infusoria *Tetrahymena pyriformis* by Gundersen and Thompson (1985). Identified in bacterial and fungal microorganisms by Tsavkelova et al. (2000)	In 1944, found in *Hermidium alipes* by Buelow and Gisvold	Discovered in 1950–1952 by pharmacologists Arvid Carlsson, Nils-Åke Hillarp and von Euler in Sweden
Norepinephrine (noradrenaline)	Identified in microorganisms by Tsavkelova et al. (2000)	In 1956–1958 found in banana fruits in Sweden laboratories organized by Waalkes and Udenfriend	Isolated from adrenal gland extracts of animals in 1897–1898 by John J. Abel
Epinephrine (adrenaline)		In 1972 found in leaves of banana *Musa* by Askar et al. (1972)	Isolated from adrenal gland extracts of animals in 1895 by Polish physiologist Napoleon Cybulski and in 1897 by American John J. Abel
Serotonin	Found in 1986 by Hsu with co-workers in many bacteria	Found in banana fruits (*Musa*) by Bowden et al. (1954)	Discovered by Erspamer in 1940 and Rapport et al. in 1948
Histamine	Found in ergot fungi *Claviceps purpurea* in 1910 by Barger, Dale and Kutscher	Observed in higher plants by Werle and Raub in 1948	In 1919 American John Jacob Abel isolated histamine from pituitary extract of animals

Sources: Fluck and Jaffe (1974), Kruk and Pycock (1990), Roshchina (1991, 2001a), Kuklin and Conger (1995), Oleskin (2007), Kulma and Szopa (2007), Shishov (2010)

Table 2.2 Level of neurotransmitters in living organisms

Neurotransmitter	In microorganisms μg/g^{-1} of fresh mass or *μmoles/L or **μg/billion of cells	In plants μg/g^{-1} of fresh mass	In animals μg/g^{-1} of fresh mass or *nM/L or **nm/day
Acetylcholine	3.0–6.6	0.1–547	0.326–65,200 (0.15–0.2 in brain,)
Dopamine	0.45–2.13*	1–4000	<0. 888*
			1214–2425**
Norepinephrine (noradrenaline)	0.21–1.87*	0.1–6760	0.615–3.23*
			20–240**
Epinephrine (adrenaline)	No data	0.22–3833	1.9–2.46*
			30–80**
Serotonin	0.11–50,000**	0.0017–4000	0.21–0.96
Histamine	0.01–3.75	1 (1,34—pain reaction for human)	0.5–100

Sources: Fernstrom and Wurtman (1971), Kruk and Pycock (1990), Hsu et al. (1986), Roshchina (1991, 2001a), Oleskin et al. (1998a, b), Tsavkelova et al. (2000)

extracts which were used as medicinal preparations. The twentieth century was the epoch for the discovery of neurotransmitters, mainly by pharmacologists and animal physiologists related to medicine. The roles of the catecholamine compounds in plants and microorganisms became a subject of interest only after 50–70 years of the twentieth century. Pioneering studies included the investigations of Jaffe, Fluck, Riov, Stephenson, Rowatt, Girvin, and Marquardt (see references in monograph of Roshchina 2001a for more detail).

As can be seen from Table 2.2, the concentration range of the neurotransmitter compounds is similar for all three kingdoms of living organisms, although some organs and specialized cells of multicellular organisms may be enriched in these compounds. Over 50 years ago, Soviet physiologist Koshtoyantz (1963) presented a hypothesis that the neurotransmitters are unique to all animal cells independent of their position on the evolutionary tree; this view has been confirmed experimentally, and has been described in several monographs (Buznikov 1967, 1987, 1990) and papers (Buznikov et al. 1996; Buznikov 2007). The presence of the neurotransmitters in animals has now been confirmed for all kingdoms—from Protozoa to Mammalia. As for bacterial cells, no more than 10–12 species have so far been characterized as containing acetylcholine, catecholamines, and serotonin, although histamine has been found in most species of prokaryotes. The issue of mammalian-type hormones in microorganisms has also been considered (Lenard 1992).

2.2.1.1 Acetylcholine

In animals, acetylcholine and/or the synthesizing enzyme choline acetyltransferase have been demonstrated in epithelial (airways, alimentary tract, urogenital tract, epidermis), mesothelial (pleura, pericardium), endothelial, muscle and immune

cells, mainly in granulocytes, lymphocytes, macrophages, and mast cells (see review of Wessler et al. 2001). Classical investigations provided early evidence to the presence of neurotransmitters and related enzymes in both microbial and plant cells.

Acetylcholine has been well-recognized as a component of bacteria (its production was discovered in a strain of *Lactobacillus plantarum*) (Stephenson et al. 1947; Rowatt 1948; Girvin and Stevenson 1954; Marquardt and Falk 1957; Marquardt and Spitznagel 1959). The first report, showing acetylcholine production in bacterium strains, was from *L. plantarum* and. *L. odontolyticus* (Stephenson et al. 1947). Approximately 5 mg acetylcholine/mg dry wt. cells/h was formed if the bacteria were grown both in vegetable juice and washed cells. Cell free enzyme(s) participating in the acetylcholine synthesis were also first found in *L. plantarum* (Girvin and Stevenson 1954).

Acetylcholine has also been found in Protozoa (Janakidevi et al. 1966a, b). Corrado et al. (2001) showed the synthesis of the molecular acetylcholine during the developmental cycle of *Paramecium primaurelia*. This neurotransmitter has a negative modulating effect on cellular conjugation. But in these unicellular organisms, the presence of functionally related nicotinic and muscarinic receptors and a lytic enzyme acetylcholinesterase has been established. Moreover, the authors demonstrate (using immunocytochemical and histochemical methods) that the activity of enzyme choline acetyltransferase, which catalyzed acetylcholine synthesis, was located on the surface membrane of mating-competent cells and of mature, but not non-mating-competent *P. primaurelia* cells.

In the plant kingdom, acetylcholine is found in 65 species belonging to 33 different families (Roshchina 1991, 2001a; Wessler et al. 2001; Murch 2006). Acetylcholine was synthesized not only in a free form, but also in a conjugated form such as, for example, cholinic esters with plant auxins (Fluck et al. 2000). Acetylcholine is particularly abundant in secretory cells of common nettle stinging hairs, where its concentration reaches 10^{-1} M or 120–180 nmol/g of fresh mass. Together with the histamine contained in the secretion, acetylcholine may provoke a pain response and the formation of blisters when the plant comes into contact with human skin. Kawashima et al. (2007) have attempted to compare the concentration of the neurotransmitter acetylcholine in a wide variety of sources using the same experimental conditions, which involved a radioimmunoassay with high specificity and sensitivity (1 pg/tube). Kawashima et al. (2007) measured the acetylcholine content in samples from the bacteria, archaea, and eukaryote domains of the universal phylogenetic tree. The authors compared the concentrations in different groups of bacteria (*Bacillus subtilis*), archaea (*Thermococcus kodakaraensis* KOD1), fungi (shiitake mushroom and yeast), plants (bamboo shoot and fern), and animals (bloodworm and lugworm). The levels of acetylcholine varied considerably, however, the highest acetylcholine content was detected in the top portion of the bamboo shoot (2.9 mmol/g), which contained about 80 times of that found in rat brain. Various levels of acetylcholine-synthesizing activity were also detected in extracts from the cells tested, which contained a choline acetyltransferase-like enzyme (sensitive to bromoacetylcholine, a selective inhibitor of choline acetyltransferase). The enzyme activity was found in *T. kodakaraensis* KOD1 (15 %), bamboo shoot (91 %), shiitake mushroom (51 %), bloodworm (91 %), and lug-

worm (81 %). Taken together, these findings demonstrate the ubiquitous expression of acetylcholine and acetylcholine-synthesizing activities among life forms without nervous systems, and support the notion that acetylcholine has been expressed and may be active as a local mediator and modulator of physiological functions since the beginning of life.

2.2.1.2 Catecholamines

In unicellular organisms, biogenic amines are also synthesized. The large amounts of dopamine accumulated by cells of infusoria *Tetrahymena pyriformis* strain NT-1 and that which was secreted into their growth medium were found to depend primarily upon the extracellular, nonenzymatic conversion of tyrosine to L-dihydroxyphenylalanine (Gundersen and Thompson 1985). Recently, the catecholamines norepinephrine and dopamine have been identified in microorganisms by high-performance liquid chromatography by Tsavkelova et al. (2000). Dopamine in concentrations of 0.45–2.13 mmol/L was found in the biomass of bacteria *Bacillus cereus*, *B. mycoides*, *B. subtilis*, *Proteus vulgaris*, *Serratia marcescens*, *S. aureus*, and *E. coli*, but was absent in the fungi *Saccharomyces cerevisiae*, *Penicillum chrysogenum*, and *Zoogloea ramigera*. Norepinephrine was found (0.21–1.87 mmol/L) in the bacteria *B. mycoides*, *B. subtilis*, *P. vulgaris*, and *S. marcescens* as well as in fungi such as *S. cerevisiae* (0.21 mmol/L) and *P. chrysogenum* (21.1 mmol/L). It is especially interesting that in many cases, the content of catecholamines in microorganisms is higher than in animals. For example in human, blood norepinephrine is found to be about 0.04 mmol/L (Kruk and Pycock 1990). Moreover, it was demonstrated that bacteria, in particular *B. subtilis*, may release norepinephrine and dopamine out of the cell and, perhaps participate in intercellular communication both in microorganism–microorganism and bacteria–host contexts.

In plants, catecholamines have been found in 28 species of 18 plant families (Roshchina 1991, 2001a; Kuklin and Conger 1995; Kulma and Szopa 2007). The amount of dopamine found varies during plant development (Kamo and Mahlberg 1984), and sharply increases during stress (Swiedrych et al. 2004). Of particular note is the finding that increased amounts of dopamine (1–4 mg/g fresh mass) are found in flowers and fruits, in particular in *Araceae* species (Ponchet et al. 1982). Derivatives of dopamine are also known in plants, for example dopamine-betaxanthin in *Portulaca oleracea* (Gandía-Herrero et al. 2009). This demonstrates the important role of the catecholamines as neurotransmitters in fertilization as well as in fruit and seed development.

High concentrations of the amines reveal the stressed state of the animal organism, including a diseased state (Gruchow 1979). The same approach in the understanding of stress agents may also be applied to plants (Swiedrych et al. 2004). Concentrations of catecholamines differ among plant species, and in some cases increased under unfavorable conditions such as ozone treatment (Roshchina and Yashin 2014; Roshchina et al. 2015a).

2.2.1.3 Serotonin

Some microorganisms living within parasitic nematodes are also able to synthesize serotonin (Hsu et al. 1986). In the bacterial flora of the ascarid *Ascaris suum*, mainly facultative anaerobes (17 species) produced and excreted serotonin into the culture medium of up to 14.32–500.00 mg/g of fresh mass for *Corynebacterium* sp. (in the tissues of the helminth itself only 0.25 mg serotonin per g fresh mass). The concentration of serotonin, in terms of mg serotonin/10^9 cells for different cultures of microorganisms isolated from helminthes is as follows: *Klebsiella pneumoniae* 8.15, *Aeromonas* 26.71, *Citrobacter* 0.58, *Corynebacterium* sp. 14.32–500.00, *Enterobacter agglomerans* 2.93, *Shigella* 1.04, *Achromobacter xylosoxidans* 1.66, *Chromobacterium* 3.67, *Achromobacter* 0.15, *Acinetobacter* 11.79, *Streptococcus* 37.52, *Listeria monocytogenes* 4.71, and *E. coli* 3.33. Serotonin has also been found in the yeast *Candida guilliermondii* and bacterium *Enterococcus faecalis* (Fraikin et al. 1989; Belenikina et al. 1991; Strakhovskaya et al. 1991, 1993). In 1998, Oleskin et al. also established the presence of serotonin in the phototrophic bacterium *Rhodospirillum rubrum* (1 mg/billion of cells ~3–12,500 mg/g of fresh mass) as well as in non-phototrophic bacteria *Streptococcus faecalis* and *E. coli* (50 and 3.3 mg/billion of cells, respectfully). Serotonin was also found in *E. coli* and *Bacillus cereus* (Shishov et al. 2009). The inhibitor of tryptophan hydroxylase, *N*-chlorophenylalanine, affects the growth of the yeast *Candida guilliermondii*, but not the development of the bacterium *E. coli*. This suggests that in the latter case, there is an alternative pathway to that found in animals (Oleskin et al. 1998a, b), which is unique (Roshchina 1991, 2001a) to plants: tryptophan → tryptamine → serotonin.

In plants, serotonin is found in 42 species of 20 plant families (Roshchina 1991, 2001a; Ramakrishna et al. 2011). Besides free serotonin, conjugated serotonins such as *N*-feruloylserotonin, *N*-(*p*-coumaroyl) serotonin, and *N*-(*p*-coumaroyl) serotonin mono-β-D-glucopyranoside have been isolated from safflower *Carthamus tinctorius* L. seed. In various plants, serotonin conjugated to form phenolic compounds via thioester linkages during the synthesis of hydroxycinnamic acid amides, including *p*-coumaroylserotonin, feruloylserotonin, and *p*-coumaroyltyramine (Ly et al. 2008). It should be noted that serotonin in animals (such as rats) may exist in complexes with heparin that prevents the aggregation of thrombocytes (Kondashevskaya et al. 1996).

2.2.1.4 Histamine

Histamine was initially discovered in the ergot fungus *Claviceps purpurea* (Table 2.1) and, subsequently, in many bacterial and plant cells by Werle and coauthors (1948, 1949). Since then, it has also been observed in many types of foods as the result of microbial activity. Histamine is one of the biogenic amines formed mainly by microbial decarboxylation of amino acids in numerous foods, including fish, cheese, wine, and fermented products. A number of microorganisms can

produce histamine. In particular, bacteria such as *Morganella morganii*, *Proteus* sp., and *Klebsiella* sp. are considered strong histamine formers in fish (Ekici and Coskun 2002; Ekici et al. 2006). Fernández et al. (2006) summarized the data on the toxicity of histamine in food. Histamine poisoning is the most common food borne problem caused by biogenic amines. At non-toxic doses, histamine can cause symptoms such as diarrhoea, hypotension, headache, pruritus, and flushes. Just 75 mg of histamine, a quantity commonly present in normal meals, can induce symptoms in the majority. One separate problem concerns the histamine formed by microorganisms in animal pathogenesis. Gram-negative bacterial species such as *Branhamella catarrhalis*, *Haemophilus parainfluenzae*, and *Pseudomonas aeruginosa* have been demonstrated to synthesize clinically relevant amounts of histamine in vitro that implicate the bacterial production of histamine *in situ* as an additional aggravating factor in acute chronic bronchitis, cystic fibrosis, and pneumonia. Histamine may also increase the virulence of these bacterial species, unlike some Gram-positive species such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (Devalia et al. 1989). Among "non-pathogenic" species, only the *Enterobacteriaceae* were found to form histamine in significant concentrations.

Significant amounts of histamine have also been observed in higher plants, initially by Werle and Raub in 1948, and subsequently described for 49 plant species belonging to 28 families ranging from basidiomycetes (now taxonomically related to Fungi) to angiosperms (Roshchina 1991, 2001a). Besides histamine itself, its derivatives *N*-acetylhistamine, *N*, *N*-dimethylhistamine, and feruloylhistamine are also found in plants. Especially high levels are observed in species of the family Urticaceae that could be one of the taxonomic classification signs. The Brazilian stinging shrub *Jatropha urens* (family Euphorbiaceae) contains 1250 mg histamine per 1000 hairs. The presence of histamine in stinging hairs is a protective mechanism that provides order to frighten off predatory animals by inducing burns, pain, and allergic reactions. Under stress conditions, a sharp increase of histamine is observed in plants, as well as in animals. Ekici and Coskun (2002) have determined the histamine content of some commercial vegetable pickles at the range of 16.54 and 74.91 mg/kg (average 30.73 mg/kg). The maximum value (74.91 mg/kg) was obtained from a sample of hot pepper pickles. The amount of histamine varies according to the phase of plant development. For example in the marine red algae *Furcellaria lumbricalis* (Huds.) Lamour, the occurrence of histamine was from 60 to 500 mg/g fresh mass observed in both non-fertile fronds and sexual-expressed parts, in all regions of the thallus of male, female, and tetrasporophyte (Barwell 1979, 1989). The amount of histamine (in mg/g fresh mass) in the male plant was 90–490 (sometimes up to 1100), in the female plant 60–120, and in asexual tetrasporophyte 100–500. Especially enriched were the neurotransmitter cells of male plants, as the ramuli were approximately five times higher in histamine than female and asexual plants. Additionally, the concentration of histamine at high salt concentration can also be increased (Roshchina 1991; Roshchina and Yashin 2014).

2.2.1.5 Location and Transport of Neurotransmitters Within Cell

According to classical publications based on the data of electron microscopic histochemistry, within the animal cells, neurotransmitters are located mainly in secretory vesicles. In microbial systems localization of catecholamines on the bacterial cell surface was confirmed by Western blot and immunofluorescence microscopy using mussel-inspired catecholamine yielded sticky *E. coli* as a new type of an engineered microbe for the study of cell-to-cell communication systems (Park et al. 2014). Mechanisms of the intercellular and extracellular transport in non-nervous systems appear to be similar with those known for animal cells. Neurotransmitters are supposed to be released into the extracellular synaptic chink (cleft) with the aid of acidic glycoproteins (~70 kDa) called vesicular monoamine transporters (Wimalasena 2011; Lawal and Krantz 2013). The sequence of neurotransmitter release of out the cell is here briefly described: (1) A hydrogen atom from the inside of the vesicle binds, inducing a conformational change in the transporter. The conformational change induced by the hydrogen atom binding enables the monoamine binding to the active transport site. (2) A second hydrogen atom binds from inside the vesicle to the transporter inducing another change. (3) The monoamine is released inside the vesicle and the two hydrogen atoms are released into the cytosol and the transport process starts over again. Moreover, the transporters are believed to be similar in bacteria to those also found in both plant and human organisms.

Localization of acetylcholine in fungal or plant cells is usually determined based on the presence of cholinesterase, an enzyme that catalyzes the decomposition of the neurotransmitter. As observed in the monograph of Roshchina (2001a), in the above mentioned cells, the enzyme has been found in the cell wall, the exine of spores and plasmalemma as well as in the nucleus and chloroplasts. In maize, acetylcholinesterase is mostly localized to the vascular bundles including the endodermis and epidermis in coleoptile nodes as well as in the mesocotyls of maize seedlings (Yamamoto and Momonoki 2012).

The location and secretions of biogenic amines is difficult to pinpoint in living, individual cells without specialized methods of identification. This difficulty in identification has been solved in animal cells via the use of molecular probes and glyoxylic acid as a reagent for catecholamines (Markova et al. 1985) and *ortho*-phthalic aldehyde as a reagent for histamine (Cross et al. 1971). Today, such histochemical methods are preferred for the microanalysis of plant single cells, as these techniques do not damage the cells and prevent the premature release of proteins, amino acids and other amines by treating with trichloroacetic acid or hydrochloric acid with or without column chromatography (Fig. 2.1). Glyoxylic acid and *ortho*-phthalic aldehyde were used to analyze the plant microspores such as vegetative microspores of *Equisetum arvense* and pollen of 25 plant species using microspectrofluorimetry and laser-scanning confocal microscopy (Roshchina et al. 2012,

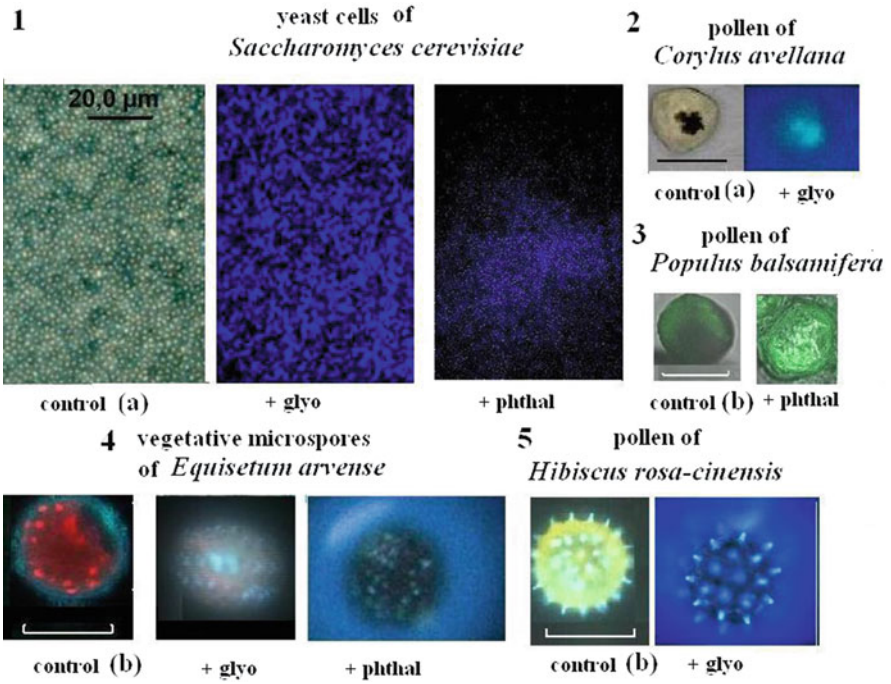

Fig. 2.1 The fluorescence images of yeast cells (**1**) and plant microspores (**2**–**5**) stained with glyoxylic acid (glyo) or *o*-phthalic aldehyde (phthal). *Bars* **1**–**4** = 20 µm, 5–100 µm. Controls **1** and **2** (*a*)—images in transmitted (transmission) light without treatment (weak fluorescence for photo), controls **3**–**5** (*b*)—autofluorescence without treatment. Excitation of emission by UV-light (360–380 nm) of luminescent microscope Leica DM 6000B. Within cells of plant vegetative or generative (pollen) microspores stained the fluorescing DNA-containing organelles such as nuclei (**2**, **3**) or nucleus and chloroplasts (**4**) are seen while on the surface of **5**—small spikes after treatment with *o*-phthalic aldehyde. The emitted excretions are also observed as holo form

2014; Roshchina and Yashin 2014). The presence of catecholamines and histamine has been found not only in secretions, but in DNA-containing organelles—nuclei and chloroplasts. Earlier, Zhirnova et al. (2007) revealed the location of histamine in the nuclei of human skin cells. Exogenous biogenic amines were also demonstrated to interact with isolated nuclei and chloroplasts (Roshchina 1990b, 1991, 2001a; Roshchina et al. 2015b). To study the single intact cells as model systems, it is necessary to use small amounts of material in order to avoid damage to cell structures.

Immunolocalization of serotonin in different tissues of *Coffea canephora* has revealed that serotonin is localized in the vascular tissues of stems, roots, and somatic embryos, as well as in endocarps (husks) of immature fruits (Ramakrishna et al. 2012a, b).

2.2.2 *Neurotransmitters as Toxicants: Ecologic, Medical, and Economic Aspects*

With the discovery of the presence of neurotransmitter substances not only in animals, but also in plants and microorganisms, medical and economic interest has increased substantially within the twenty-first century. Interest lies in concern of interactions between organisms in biocenosis that includes various non-neuronal functions of neurotransmitters in animals but also concerns the regulation of growth and morphogenesis in microorganisms, animals, and plants. Interest also includes protection in the adaptation to environmental changes as well as other functions. This development contributes benefits to various aspects of medicine insofar that some serotonin and dopamine-containing fruits may protect against Alzheimer's disease (see Sect. 2.4). An economic standpoint illustrates the economic concern of contamination of food products by stress metabolites produced by plants or animals. Bacterial or fungal microorganisms are known to influence the quality and freshness of food products. Such microorganisms may contaminate such foods through their production and secretion of catecholamines or histamine.

High concentrations of biogenic amines in foodstuffs and beverages can induce a range of toxicological effects (Fernández et al. 2006). Significant attention is needed to control the histamine levels in foods (Bodmer et al. 1999). Histamine poisoning is the most common food-borne problem. Aside from the compounds naturally found in vegetables and fruits as well as those formed as a result of the fermentation of cheese, wine, and sauerkraut, biogenic amines also play an essential role in the metabolism of the histamine-forming bacteria present in foods (Kung et al. 2007). Flushing of the face and neck are symptoms of histamine intoxication, followed by an intense, throbbing headache. Other symptoms include dizziness, itching, faintness, burning of the mouth and throat, and the inability to swallow. Taylor et al. (1978) reported that ingestion of 70–1000 mg of histamine in a single meal is necessary to elicit any symptoms of toxicity. A level of histamine exceeding 10 mg/100 g of fresh weight is associated with poor product quality indicative of microbial spoilage, with levels of 200 mg histamine per kg of food product accepted as a toxic indicator for fish, and 10 mg/kg for wines, whereas for hot pepper pickles values are generally below the level of 1000 mg/kg.

On average, histamine content in food of approximately 30 mg/kg can be considered the minimal level for clinical symptoms of toxicity (Ekici and Coskun 2002). These toxicological problems are particularly severe in individuals who, for whatever reason, are deficient in diamine oxidase, the histamine-degrading enzyme. At non-toxic doses, histamine can cause intolerance symptoms such as diarrhea, hypotension, headache, and flushing. Just 75 mg of histamine, a quantity commonly present in normal meals, can induce symptoms in the majority of healthy persons with no history of histamine intolerance. Serotonin also may interact with *Candida albicans* and regulate the fungal organism's virulence (Mayr et al. 2005)

The amount of neurotransmitters in cellular secretions can be increased following stressful stimuli, including certain interactions with other organisms. Large

amounts of dopamine are usually secreted by cells of infusoria *Tetrahymena pyriformis* into their growth medium (Gundersen and Thompson 1985). This release of dopamine is especially important during infection, when the animal or plant accumulates some of the neurotransmitters, while pathogens release neurotransmitters (Romanovskaya and Popenenkova 1971). On north-eastern Pacific coasts, the alga *Ulvaria obscura* produces large amounts of dopamine (van Alstyne et al. 2006). This organism, dominant in subtidal "green tide" blooms due to this anti-herbivore defense, can be harmful to marine communities, fisheries, and aquaculture facilities because the alga presence is the cause of reduced feeding by echinoderms, molluscs, and arthropods.

Dopamine constituted an average of 4.4 % of the alga's dry mass, and was responsible for the decreased feeding by sea urchins (*Strongylocentrotus droebachiensis*). Subsequent experiments demonstrated that dopamine also reduced the feeding rates of snails (*Littorina sitkana*) and isopods (*Idotea wosnesenskii*). This is the first experimental demonstration of a plant (algal) catecholamine functioning as a feeding deterrent.

Five histamine-producing bacterial strains isolated by Jaw et al. (2012) from fish meal samples producing 1.31–6.21 ppm of histamine in trypticase soy broth supplemented with 1.0 % histidine were identified as *Bacillus licheniformis* (three strains), *B. amyloliquefaciens* (one strain), and *B. subtilis* (one strain). Legal upper limit of biogenic amines as toxicants (Silla Santos 1996): histamine: 100 mg/kg food. 1000 mg/kg amine (based on histamine intoxication & amine concentration in food) is dangerous for human health. The toxic dose of biological amines depends on the individual's own sensitivity and other unique characteristics. Individuals may influence cell proliferation, regulate nucleic acid function, and protein synthesis. Products such as wine, beer, and other foods that are prepared with the yeast *S. cerevisiae*, require attention be given to microorganismal biogenic amines. Histamine intoxication can cause nausea, respiratory distress, hot flushing, heart palpitation, headache, hypertension and hypotension.

Current rapid detection methods for neurotransmitters in food and environmental samples have proved invaluable. The use of gas and liquid chromatographic methods in the detection of biogenic amines in vegetables (Ly et al. 2008), is complemented by the histochemical fluorescent method of staining with glyoxylic acid or o-phthalic aldehyde, for example, in the analysis of algae (Barwell 1979, 1989) and allergenic pollen (Roshchina et al. 2014; Roshchina and Yashin 2014). High concentrations of catecholamines or histamine released by algae may be hazardous for the development of surrounding species, including sea animals (Barwell 1979; van Alstyne et al. 2006, 2011, 2013, 2014). Catecholamine and histamine release are also of concern in eliciting allergic reaction in humans following contact with pollen (Roshchina et al. 2014).

The influence of neurotransmitters in aquatic biocenosis is both relevant and useful in marine ecology. Instances of usefulness to marine ecology include bacterial response-reaction to some diatoms (Kaeppel et al. 2012), such as the marine *Marinobacter adhaerens*, which aggregates with the diatom *Thalassiosira weissflogii* sp. nov.

2.2.3 Components of Cholinergic and Aminergic Systems

In microbial cells, components of cholinergic and aminergic systems are similar to those found in mammalian cells, including the complete biosynthetic pathway required for their synthesis (synthetases) and their catabolism (cholinesterases, aminooxidases, and others), as well as functional analogues of cholino- and aminoreceptors have been demonstrated to be present.

2.2.3.1 Choline Acetyltransferase

The enzymes choline acetyltransferases or choline acetylases (EC 2.3.1.6) participate in the synthesis of acetylcholine from choline and acetic acid (Nachmansohn and Machado 1943). A cell-free enzyme preparation with "choline acetylase" activity was present in *Lactobacillus plantarum* (Girvin and Stevenson 1954). This type of enzyme activity has also been found in many plant species (Roshchina 1991, 2001a).

2.2.3.2 Cholinesterase

Enzymes which degrade acetylcholine to choline and acetic acid are named cholinesterases and were first found in 1937 by Loewi in the hearts of amphibia. The function of acetylcholinesterase at cholinergic synapses of animals is to terminate cholinergic neurotransmission (Augustinsson 1949). However, the enzyme is expressed in tissues that are not directly innervated by cholinergic nerves. Moreover, transient expression in the brain during embryogenesis suggests that acetylcholinesterase may function in the regulation of neurite outgrowth. Overexpression of cholinesterases has also been correlated with tumorigenesis and abnormal mega-karyocytopoiesis (Small et al. 1996). Cholinesterase is also found in unicellular animals such as *Paramecium* (Corrado et al. 1999). An immunoblot analysis of the *Paramecium* enzyme revealed that the acetylcholinesterase had a molecular mass from 42 to 133 kDa, as reported for an analogous enzyme isolated from higher organisms. Structural homologies between cholinesterases and the adhesion proteins indicate that cholinesterases could also function as cell–cell or cell–substrate adhesion molecules. Abnormal expression of cholinesterases of both types has been detected around the amyloid plaques and neurofibrillary tangles in the brains of patients with Alzheimer's disease (Small et al. 1996).

As for microorganisms, Goldstein and Goldstein (1953) first described the production of cholinesterase by a strain of bacterium *Pseudomonas fluorescens* after the culture was grown with acetylcholine as the sole source of carbon. The *P. fluorescens* enzyme was inducible, mainly, by choline (not as a carbon substrate, but, perhaps, as a source of nitrogen) or by two- to threefold less by some choline esters: acetylcholine > propionylcholine = benzoylcholine > butyrylcholine > acetyl-β-methylcholine. Addition of glucose completely prevented induction of the *P. fluorescens* enzyme. The pH optimum for growth of the culture and cholinesterase activity

was 7.0, although the culture growth was higher in alkaline medium, where spontaneous hydrolysis of acetylcholine is also maximal. Choline oxidase synthesis in *P. fluorescens* has also been induced by choline. The cholinesterase of the bacterium may hydrolyze acetylcholine or propionylcholine, but to a lesser degree butyrylcholine, benzoylcholine, or acetyl-β-methylcholine. Like cholinesterase in animals, the enzyme activity in *P. fluorescens* was inhibited by neostigmine, with complete inactivation observed at high concentrations (10^{-3}–10^{-2} M) and only partly at the lower levels of 10^{-6} M. These levels of inhibition are similar to that observed in mammalian organ systems. The *P. fluorescens* protein was isolated and characterized (Goldstein 1959; Searle and Goldstein 1957, 1962; Fitch 1963a, b). Moreover, the strains of *P. fluorescens* tested exhibited preference for acetylcholine for growth promotion over choline, glycerol, glucose, succinate, betaine, and serine (Fitch 1963a). The isolated cholinesterase was inhibited by neostigmine in smaller (1000 times) concentration, than by physostigmine, but was not inhibited by diisopropylfluorophosphate (Fitch 1963b). A bell-shaped substrate saturation curve was observed, and specific activity of the 115-times purified cholinesterase was 10.5 mmol/mg protein/min. The enzyme had features both of true cholinesterase and acetylcholinesterase (Laing et al. 1967, 1969). Specific activity of the cholinesterase from *P. fluorescens* purified 40-fold by CM-50 Sephadex was up to 70 mmol/mg protein/min. The values of *Km* at pH 7.4 and 37 °C were 1.4×10^{-5} M for acetyl-choline and 2.0×10^{-5} M for propionylcholine, respectively, while butyrylcholine and benzoylcholine were not hydrolysable at all. The purified enzyme was inhibited by organophosphorus compounds and neostigmine, but not by physostigmine.

Imshenetskii et al. (1974) showed that a large variety of microorganisms may decompose acetylcholine including 31 strains of bacteria (genera *Arthrobacter* and *Pseudomonas*) and two strains of fungi (from 194 strains studied) that live in soil. Around 100–200 mg of wet biomass of active microbial strains were able to decompose 15–30 mmol of acetylcholine during a 2 h incubation, with the most active strains (50 mg of wet biomass) able to degrade up to 10 mmol/min. This active soil strain was identified as *Arthrobacter simplex* var. *cholinesterasus* var. *nov*. The amount of the decomposed acetylcholine by this microbe was 30 times higher than in other strains (*Pseudomonas fluorescens*—4 mM/h, *P. aeruginosa*—1 mM/h), while *Arthrobacter simplex* var. *cholinesterasus* var. *nov* had an activity of 300 mM/h. Actinomycetes (except two strains) and yeast had no significant cholinesterase activity. Our experiments showed a small rate of hydrolysis of acetylthiocholine and butyrylthiocholine in extracts of *Saccharomyces cerevisiae* where only 20–30 % inhibited by neostigmine or physostigmine, inhibitors of animal cholinesterase. Roshchina and Alexandrova (1991) isolated cholinesterase from the mycelium of the fungus *Aspergillus niger*. The enzyme hydrolyzed cholinic esters at rates similar with animal enzyme (K_M 7×10^{-4} M). Moreover, the enzyme has a molecular mass about 600 kDa determined by gel-filtration on Sephadex G-200 and after gel-electrophoresis with sodium dodecyl sulphate—two subunits 63 and 44 kDa. The enzyme activity was higher with acetylthiocholine, than butyrylthiocholine, and neostigmine inhibited the substrates' hydrolysis, unlike physostigmine.

Cholinesterase activity has also been found in lower groups of the plant kingdom: in extracts of Characeae algae *Nitella* by Dettbarn, in 1962 and mycelium of fungi *Physarum polycephalum* by Nakajima and Hatano in 1962, and then a series of classical papers of Jaffe and Fluck with coworkers in 1970–1975 were devoted to the observation of the enzyme in many plant species: ~118 terrestrial species and ten marine algae were identified as having cholinesterase activity (for more details see the relevant references in monographs Roshchina 1991, 2001a). The values of the enzyme activity (the substrate hydrolysis rate) in higher plants were an average of 1–900 mmol/h/g fresh weight, depending on the plant species. It was shown that Bryophytes (mosses, liverworts and hornworts) demonstrate maximal cholinesterase activity of up to 0.360 mmol/h/g fresh weight (Gupta et al. 2001). Pollen also contain the enzyme (Bednarska and Tretyn 1989; Bednarska 1992; Roshchina et al. 1994; Rejon et al. 2012). Thus, detection of cholinesterase activity could serve as an additional indicator of the presence of acetylcholine.

Recently, the identification, purification, and cloning of maize acetylcholinesterase provided the first direct evidence of the enzyme formation in plants (Sagane et al. 2005). An especially important fact is that the acetylcholinesterase distribution in seedlings is sensitive to gravity, leading to asymmetry of the enzyme distribution (Momonoki 1997). Maize acetylcholinesterase activity localized in vascular bundles the coleoptile node and mesophyll seedlings is enhanced through a post-translational modification response to heat stress (Yamamoto and Momonoki 2012). The gene purportedly encoding for acetylcholinesterase was cloned from maize (Muralidharan et al. 2013). The *Arabidopsis* protein encoded by the gene exhibited lipase activity with preference for long chain substrates but did not hydrolyze choline esters. In several edible fruits and vegetables, members of the Solanaceae family (potato, eggplant and tomato), acetylcholinesterase inhibitors have been detected; these were mainly alkaloids which induced gastrointestinal and neurological symptoms in mammalian.

Plant cholinesterases are included in several processes (Roshchina and Semenova 1990; Roshchina 2001a, b), including but not limited to: pollen germination release just after moistening of the pollen grains (Roshchina et al. 1994; Roshchina 2007), as well as within the intercellular interactions in the pollen-pistil system (Kovaleva and Roshchina 1997). Cholinesterase may prevent the binding of dopamine with its receptor and decrease the toxic effect of d-tubocurarine (antagonist of nicotinic type of cholinoreceptor) on cells of horsetail vegetative microspores (Roshchina et al. 2012). Yamamoto and co-workers (2011) postulated that acetylcholinesterase is a positive regulator of heat tolerance in plants.

2.2.3.3 Enzymes of Biogenic Amine Metabolism

The biosynthetic pathway of biogenic amines includes the decarboxylation and hydroxylation of corresponding amino acids, in particular phenylalanine for the catecholamines, tryptophan for serotonin, and histidine for histamine (Lawrence 2004). Phenylalanine, precursor of dopamine, norepinephrine and epinephrine, is

first hydroxylated, transforming to tyrosine and then to dihydroxyphenylalanine (DOPA). These processes are catalyzed by phenylalanine hydroxylase or phenylalanine monoxidase and tyrosine hydroxylase or tyrosine-3-monoxidase. Dopamine, an immediate precursor of norepinephrine and epinephrine, arises from DOPA through decarboxylation by means of the enzyme decarboxylase dioxyphenylalanine and the decarboxylase of aromatic amino acids (EC 4.1.1.26). Another route of tyrosine transformation is via decarboxylation, when it transforms to tyramine, and then by hydroxylation with the participation of tyramine hydroxylase into dopamine, which is then oxidized to norepinephrine by the copper-containing enzyme-β-hydroxylase 3,4-dioxyphenylethylamine. Then, under the influence of the transmethylase of phenylethanolamines, the formation of epinephrine takes place.

In the catabolism of catecholamines, aminooxidases participate as in the oxidative deamination of the catecholamines to metanephrine, normetanephrine, vanillic aldehyde, dehydroxymandelic and vanillic acids. For microorganisms, this metabolic process has not yet been studied. In plants, diamineoxidases play the main role in catecholamine metabolism, unlike animals that use monoaminooxidases for this purpose (Roshchina 2001a). As for catecholamine-*O*-methyltransferases, they are present in all animal tissues, and especially active in nervous cells. In plants, the catecholamine-*O*-methyltransferases pathway is also possible because the last three compounds are ordinary products of plant metabolism (Kuklin and Conger 1995; Roshchina 2001a; Kulma and Szopa 2007). Besides the abovementioned pathways of metabolism, catecholamines are oxidized by oxygen in air, forming oxidized products—red pigments aminochromes and black-brown pigments melanines which are polymers of indole (found both in plant and animals). The mechanism of oxidation is connected with the generation of superoxide radicals. Prevention of the oxidation of dopamine by superoxide dismutase confirms this mechanism. Enzymatic oxidation of catecholamines to melanines by polyphenol oxidase has been demonstrated (Roshchina 2001a). The abovementioned enzymes are found only in animals and plants. There is little data for catecholamine oxidation in microorganisms, although monoaminooxidase activity in mycobacteria (Pershin and Nesvadba 1963) and *E. coli* (Takenaka et al. 1997) has been found.

Serotonin is synthesized in plants and animals from tryptophan formed by the shikimate pathway, which has also been proposed for microorganisms (Oleskin et al. 1998a, b; Oleskin 2007). This process proceeds by two pathways: either via 5-hydroxytryptophan or tryptamine formation, or the first step of serotonin biosynthesis via decarboxylation of tryptophan, which then transforms in plants to tryptamine by action of the enzyme tryptophan decarboxylase (EC 4.1.1.27), or by the decarboxylation of aromatic amino acids (EC 4.1.1.26/27). Then, tryptamine is transformed to serotonin by hydroxylation with participation of the enzymes tryptamine-5-hydroxylase or 1-tryptophan-5-hydroxylase (EC 1.14.16.4). Hydroxylation of tryptophan leads to the formation of 5-oxytryptophan in the presence of tryptophan-5-hydroxylase (EC1.14.16.4). At the next stage, 5-oxytryptophan is decarboxylated by the decarboxylation of aromatic acids to yield serotonin. Tryptamine 5-hydroxylase, which converts tryptamine into serotonin and is common in animals, was also found as a soluble enzyme that had maximal activity in rice roots

(Kang et al. 2007). The tissues of rice seedlings grown in the presence of tryptamine exhibited a dose-dependent increase in serotonin in parallel with enhanced enzyme activity. However, no significant increase in serotonin was observed in rice tissues grown in the presence of tryptophan, suggesting that tryptamine is a bottleneck intermediate substrate for serotonin synthesis. If we compare the enzymes from the different kingdoms, we can see more similarity. In particular, in the plant genus *Arabidopsis*, there is a homolog to part of a DNA binding complex corresponding to the animal tyrosine and tryptophan hydroxylases (Lu et al. 1992). Fujiwara et al. (2010) have found that unlike tryptophan hydroxylase of animals, an analogous enzyme in plants is a cytochrome P450 located in the membranes of the endoplasmic reticulum. The enzyme was not expressed in the bacteria *E. coli*, but may be expressed here via special genetic construction with recombinant genes (Park et al. 2011). Aminooxidases of biogenic amines may differ in microorganisms in relation to substrate specificity, in particular for the bacterium *Methanosarcina barkeri* and infusoria *Tetrahymena pyriformis* (Yagodina et al. 2000). Both studied enzymes can deaminate serotonin, but not histamine. The existence of one active centre for substrate binding is postulated as the aminooxidase of the bacterium, while several centres are thought to exist in the Infusorians.

For all living organisms, the biosynthesis pathway of histamine includes histidine decarboxylase which participates in the decarboxylation of histidine (Roshchina 2001a; Boron and Boulpaep 2005; Martín et al. 2005). The gene encoding histidine decarboxylase (*hdcA*) has been identified in different Gram-positive bacteria (Martín et al. 2005). Histidine decarboxylase used to be part of a cluster that included a gene of unknown function (*hdcB*) and a histidine–histamine antiporter gene (*hdcC*) in *Pediococcus parvulus* 276 and *Lactobacillus hilgardii* 321 has been identified (Landete et al. 2005). Catabolism of histamine occurs also via methylation or acetylation in the presence of histamine-*N*-methyltransferase, or histamine-N-acetyltransferase, and genes coding of the enzymes have been found in bacteria, plants, and animals (Iyer et al. 2004).

2.2.3.4 Recognition of Neurotransmitters

Appreciation of the presence and role of neurotransmitters in microbial cells was previously considered mainly in the context of receptors specific to the neurotransmitter compounds (Adler 1969), with particular understanding gained from concepts of neurotransmitter reception in animals. A major method by which organisms may be studied is the use of pharmacological assays wherein the neurotransmitter of a cellular reaction is analyzed via the use of agonists and antagonists to that neurotransmitter. All investigations concerning non-neuronal (non-synaptic) systems have a fundamental similarity to traditional studies undertaken for the nerve cell.

For acetylcholine, there are two types of acetylcholine receptors: nicotinic (receptors that respond to nicotine) and muscarinic (receptors that are sensitive to muscarine). Corrado et al. (2001) showed the presence of functionally related nicotinic and muscarinic receptors and the lytic enzyme acetylcholinesterase in the

unicellular animal *Paramecium primaurelia*. In plants, the presence of similar receptors has also been shown (Roshchina 2001a). Recently, it was established that muscarinic and nicotinic acetylcholine receptors are involved in the regulation of stomata function—the opening and closing movement—in the plants *Vicia faba* and *Pisum sativum* (Wang et al. 1998, 1999a, 2000). Leng et al. (2000) showed the regulation role of acetylcholine and its antagonists in inward rectified K^+ channels from *Vicia faba* guard cells. Location of the muscarinic receptor was shown in plasmatic membrane and chloroplast membranes (Meng et al. 2001), and cholinesterase activity was found in the cells (Wang et al. 1999b). The germination of plant microspores such as vegetative microspores of horsetail *Equisetum arvense* or pollen (generative microspores) of knight's star *Hippeastrum hybridum* was blocked by the antagonists of acetylcholine, which are linked with nicotinic cholinoreceptors and Na^+/K^+ ion channels (Roshchina and Vikhlyantsev 2009). The nicotinic cholinoreceptors were cytochemically identified in the single-cell amoebae *Dictyostelium discoideum*, slugs, and spores, however, proteins immunologically-related to the muscarinic receptors were not present in the spores (Amaroli et al. 2003). Interestingly, nicotine and acetylcholine as the ligands of human nicotinic cholinoreceptors in culture of epithelial cells HEp-2 may stimulate the growth of *Chlamydia pneumoniae* (Yamaguchi et al. 2003).

The receptors for biogenic amines which are unique to animals are known as dopamine receptors, adrenoreceptors, and serotonin and histamine receptors. Similar receptors were observed in bacteria (Lyte and Ernst 1993; Lyte 2004; Freestone et al. 2007) and in plant cells (see monographs Roshchina 1991, 2001a). Alpha and beta adrenergic-like receptors may be involved in catecholamine-induced growth of Gram-negative bacteria (Lyte and Ernst 1993). In particular, Freestone et al. (2007) showed the blockade of catecholamine-induced growth of *E. coli*, *Salmonella enterica*, and *Yersinia enterocolitica* by adrenergic and dopaminergic receptor antagonists. In plants, adrenoreceptors participate in cytoplasm movement, ion permeability, and membrane potential, in flowering of *Lemna paucicostata*, photophosphorylation, as well as seeds, vegetative microspores' and pollen germination (Roshchina 1991, 2001a; Baburina et al. 2000; Kulma and Szopa 2007). Serotonin- and histamine-sensitive receptors in plants regulate the seed, pollen, and vegetative microspores' germination (Roshchina 1991, 2001a, 2004, 2005a). Shmukler et al. (2007) discussed earlier hypotheses of protosynapse for low-complexity organized animals and embryos of high-complexity organized animals, where the distribution of membrane serotonin receptors is restricted to the period of blastomere formation during cleavage and localized within the area of interblastomere contact. The hypothesis was based on their experiments where the membrane currents of the *Paracentrotus lividus* early embryos were registered after local application of serotonin drugs via special micropipette. Receptors of neurotransmitters may be linked with ion channels. Moreover, some domains of the ion channels appear connected to the cytoskeleton, in particular with actin (Cantiello 1997), and so the received chemosignal is likely to spread to the organelles via actomyosin filaments (Roshchina 2005a, 2006a, b).

Aspects of newer investigations of receptors are concerned with recombinant systems. For example, the recombinant mouse brain serotonin receptor (5HT1c) was used to study the response of plant cells and oocytes to a stress signal activated by the serotonin–serotonin receptor interaction and associated Ca^{2+} flux (Beljelarskaya and Sutton 2003). Based on plant expression vectors, recombinant constructs were obtained to direct production of 5HT1c fused with the green fluorescent protein in plant cells. The mRNAs for hybrid proteins were synthesized in an in vitro transcription system. The expression and function of the hybrid protein and the function of the associated ion channels were electrophysiologically studied in *Xenopus laevis* oocytes injected with the hybrid mRNA. The hybrid protein was functional and changed the operation of the Ca^{2+} channel in oocytes. To study the expression of the hybrid constructs in plant cells, the in vitro transcription product was inoculated in tobacco leaves, which thereupon fluoresced.

2.3 Perspectives on the Universal Functions of Neurotransmitters (Biomediators)

The presence of neurotransmitters in organisms in general leads us to the problem of information transmission within and between living cells. Like the genetic code, a common class of mediator-type molecules within all living organisms indicates that the mechanism of reaction-response (communication) appears to have a common foundation in the form of universal, or universally-recognized, chemical signals. The compounds acetylcholine and biogenic amines categorized as neurotransmitters, besides having specialized mediator function in organisms with nervous systems, also play other roles, not only in animals, but also in microorganisms and plants. As such, one could call the compounds "biomediators," rather than "neurotransmitters" or "neuromediators" (Roshchina 1989, 1991).

2.3.1 Functions of Neurotransmitters on Different Evolutionary Steps

The function of compounds named neurotransmitters originates from simple chemotaxis and chemosignaling of microbial cells and leads to intercellular communication (Fig. 2.2). The so-called neurotransmitters may regulate (as hormones) growth and development of other unicellular organisms, and be attractants or repellents for them. In higher concentrations the same substances also play a role in defense (i.e. such as in the fight or flight response) or, in some cases, contribute to creating unique cultural foundations for food and cuisine. The following step in evolution includes the development of interactive relationships (parasitic, symbiotic, or otherwise) as well as the formation of multicellular organisms that may further specialize

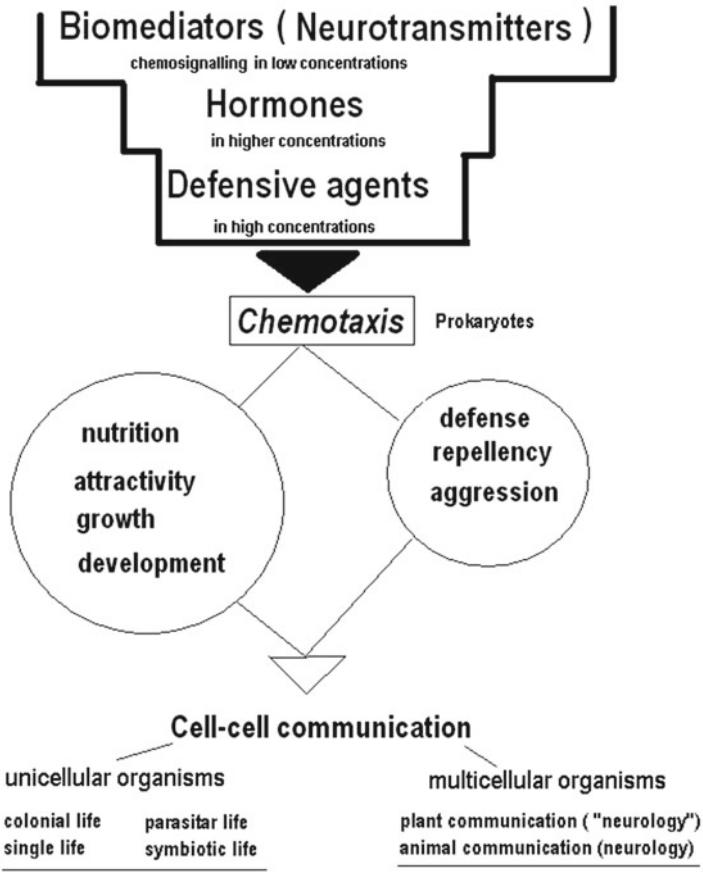

Fig. 2.2 Evolutionary development of neurotransmitter (biomediator) function

and attenuate the function of biomediators to serve unique requirements of communicative reaction-response within a larger, more complex multicellular system. This evolutionary pathway leads us to the concept of neurology as applicable to both animals and plants (Baluska et al. 2005; Brenner et al. 2006; Murch 2006; Ramakrishna et al. 2011).

In Table 2.3, the main functions of the neurotransmitters in all kingdoms are compared. The overall effect of a communication signal into a cell from an extracellular surface site or even intracellularly between cellular compartments, may occur via neurotransmitters. At the cellular level, the compounds may induce different reactions. Neurotransmitters are stored in secretory vesicles, and can be released inside or outside of the cell. Primary reaction to acetylcholine is often a change in membrane permeability for ions, while other reactions for both the neurotransmitter and biogenic amines are connected with the systems of secondary messengers—cyclic nucleotides, Ca^{2+}, inositol-3-phosphate, and others.

Table 2.3 The established functions of neurotransmitters in living organisms

Neurotransmitter	Microorganisms	Plants	Animals
Acetylcholine	Regulation of motility	Regulation of membrane permeability and other cellular reactions up to growth and development in many plant species	Regulation of cell proliferation, growth and morphogenesis. The carriage of nerve impulses across the synaptic chink, from one neuron to another of impulses across the "motor plate", from a neuron to a muscle cell, where it generates muscle contractions
	Substrate and nitrogen origin	Regulation of contractile proteins functioning	
		Action potential production	
Dopamine	Stimulation the cultural growth of *E. coli*, *Y. enterocolitica*, *S. enterica*, *S. epidermidis* etc., and the cellular aggregation and formation of colonies of *E coli*	Regulation of many cellular processes from growth and development to defence reactions	Decreases peripheral vascular resistance, increases pulse pressure and mean arterial pressure. The positive chronotropic effect produces a small increase in heart rate as well. Important for forming memories. In embryos of Vertebrata and lower animals may regulate development
	Regulation of production of some metabolites, virulence processes, bacterial motility and formation of biofilms	Regulation of contractile proteins functioning	
Norepinephrine (noradrenaline)	The similar effects for Gram-negative bacteria *E. coli*, *S. enterica,* and *Y. enterocolitica*	Regulation of many cellular processes from growth and development to defence reactions	Increases peripheral vascular resistance, pulse pressure and mean arterial pressure as well as stimulates of the thrombocytes' aggregation
		Regulation of contractile proteins functioning	
Epinephrine (adrenaline)	Similar effects on bacteria to those described for norepinephrine	Regulation of many cellular processes from ion permeability, growth and development to defence reactions	Induced vasodilation (mainly in skeletal muscle) and vasoconstriction (especially skin and viscera)
		Regulation of contractile proteins functioning	
Serotonin	Stimulation of growth of culture and cellular aggregation bacteria *Streptococcus faecalis*, yeast *Candida guilliermondii*, *E. coli K-12* and *Rhodospirillum rubrum*	Regulation of growth and development of many plant cells	Control of appetite, sleep, memory and learning, temperature regulation, mood, behaviour (including sexual and hallucinogenic), vascular function, muscle contraction, endocrine regulation, and depression. In embryos of Vertebrata and lower animals may regulate development
	Regulation membrane potential		
Histamine	Stimulation of cultural growth and cellular aggregation of *E. coli* K-12	Regulation of the growth and development at stress	Involves in many allergic reactions and increases permeability of capillaries, arterial pressure is decreased, but increases intracranial pressure that causes headache, smooth musculature of lungs is reduced, causing suffocation, causes the expansion of vessels and the reddening of the skin, the swelling of cloth
			Stimulation of the secretion of gastric juice, saliva (digestive hormone)

Sources: Anuchin et al. (2007, 2008), Buznikov (1967, 1987, 1990), Faust and Doetsch (1971), Burton et al. (2002), Freestone et al. (2007), Imshenetskii et al. (1974), Kisnieriene et al. (2012), Lyte and Ernst (1992, 1993), Lyte et al. (1997), Oleskin et al. (1998a, b), Oleskin (2007), Malikina et al. (2010), Roshchina (1991, 2001a, 2014), Roshchina and Vikhlyantsev (2012), Shishov et al. (2009), Strakhovskaya et al. (1993), Zholkevich et al. (2003), Zholkevich et al. (2007a, b)

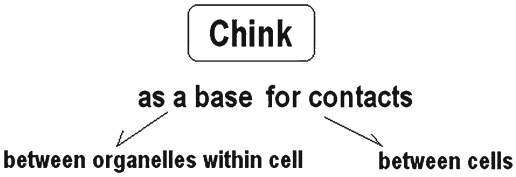

Fig. 2.3 Site, or structure, where the action of neurotransmitters may take place

A neurotransmitter functions as a chemosignal (i.e. the neurotransmitter is released from a cell and elicits a reaction in another cell) in the context of certain structures, including intercellular connections as well as intracellular environment such as between organelles within a cell. We can usually see the intercellular structural connection between plasma membranes of any cells that are in contact with one another, such as at the membranes of unicellular organisms or synaptic membranes of cells in organisms with a nervous system (Roshchina 1991, 2001a, b; Buznikov et al. 1996; Shmukler et al. 2007). As seen in Fig. 2.3, at any membrane point-of-contact either transitory or permanent, structural connections may be formed. There are junctions between endoplasmic reticulum and organelles within cells, or between different cells. Today, permanent or transitory structural-links between cells or within the cell are considered a necessary structural component in the transfer of the chemosignal.

As can be seen in Table 2.3, common cellular effects of neurotransmitters in living cells of all kingdoms include changes in membrane permeability (short-term effects) as well as the regulation of growth and development (long-term effects). The regulatory functions of neurotransmitters appear to have been conserved through evolution, effectively relating intracellular processes with unicellular populations within the environment. In this respect, the primary role of such compounds appear to be as a substrate in the acquisition of nitrogen by microorganisms (Imshenetskii et al. 1974), as well as serving as regulators in the microorganismal growth and development (Oleskin et al. 2010; Oleskin 2012).

2.3.1.1 Functions in Microorganisms

The ability of bacteria and fungi to both produce and respond to the panoply of neuroendocrine hormones that are more commonly associated with mammalian organisms is becoming increasingly recognized as playing a pivotal role in both disease pathogenesis as well as the maintenance of homeostasis (Lyte 2013). The beneficial role of probiotics enriched in such compounds has also been investigated (Lyte 2011; Freestone 2013), as well as has their possible role in soil nitrogen fixation by bacteria living in symbiosis with plants.

Acetylcholine. Early function of acetylcholine was demonstrated in its regulation of motility of the photosynthetic bacteria *Rhodospirillum rubrum* and *Thiospirillum jenense* (Faust and Doetsch 1971) as well as in non-photosynthetic bacteria, such as *Pseudomonas fluorescens* (Chet et al. 1973). Acetylcholine can be also used as

substrate for microorganisms, and regulates their development in special conditions (Imshenetskii et al. 1974).

Catecholamines. Early publications showed that the chemotactic response of *Pseudomonas fluorescens* was significantly enhanced by epinephrine, but acetylcholine, a physiological antagonist of epinephrine, inhibited bacterial chemotaxis (Chet et al. 1973). Unlike acetylcholine, catecholamines act as regulators of growth and development of many microbial cultures.

Catecholamines can regulate the growth of Gram-negative bacteria, including *E. coli* (where concentration dependent specificity was observed with response to norepinephrine > epinephrine > dopamine), *Y. enterocolitica* and *P. aeruginosa* (Lyte and Ernst 1992; Freestone et al. 1999). Dopamine also stimulates the cultural growth of *E. coli*, *Y. enterocolitica*, *S. enterica*, *S. epidermidis*, etc., and the cellular aggregation and formation of colonies of *E. coli* and *S. epidermidis* (Lyte and Ernst 1993; Neal et al. 2001; Freestone et al. 2007; Anuchin et al. 2007, 2008). Similar effects on Gram-negative bacteria *E. coli*, *S. enterica* and *Y. enterocolitica* were observed for norepinephrine (Lyte and Ernst 1992, 1993; Lyte et al. 1997; Freestone et al. 1999, 2007; Burton et al. 2002) and on *E. coli* for epinephrine (Anuchin et al. 2007; Freestone et al. 2007). Freestone et al. (2008a, b) showed that catecholamine stress hormones can significantly increase the growth of a wide range of gram negative and gram positive bacteria. Various effects of catecholamines and their agonist isoproterenol added in pathogenic bacterial cultures were observed, although all of the compounds markedly increased during bacterial growth compared to controls (Belay and Sonnenfeld 2002). Norepinephrine and dopamine had the greatest enhancing effects on growth of cultures of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, while epinephrine and isoproterenol also enhanced growth to a lesser extent. The growth of *Escherichia coli* in the presence of norepinephrine was greater than growth in the presence of the three other neurochemicals used in the study. Growth of *Staphylococcus aureus* was also enhanced in the presence of norepinephrine, but not to the same degree as was the growth of Gram-negative bacteria.

Besides growth reactions, catecholamines may regulate production of some metabolites, virulence processes, bacterial motility and formation of biofilms. Stress catecholamines (50 μM adrenaline and noradrenaline) were shown to stimulate the production of volatile sulfur compounds (mainly hydrogen sulfide H_2S) by periodontopathogenic bacteria *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, *Prevotella intermedia* and *Porphyromonas* that produce volatile sulfur compounds, the major gases responsible for bad breath (Calil et al. 2014). Bacteria can use mammalian hormones to modulate pathogenic processes that play essential roles. Catecholamines from pathogenic microflora are capable of regulating gene expression in disease development, for example it is known that the bacterium *Actinobacillus pleuropneumoniae*, an important porcine respiratory pathogen, causes significant economic loss in the global pig industry (Li et al. 2012). 158 and 105 genes were differentially expressed in the presence of epinephrine and norepinephrine, respectively. Only 18 genes were regulated by both hormones. Adhesion of the bacterium *Actinobacillus pleuropneumoniae* to host cells

was induced by norepinephrine but not by epinephrine (Li et al. 2012). This study revealed *A. pleuropneumoniae* gene expression, including those encoding virulence factors, was altered in response to both catecholamines. The differential regulation of *A. pleuropneumoniae* gene expression by the two hormones suggests that this pathogen may have multiple responsive systems for the two catecholamines. *Porphyromonas gingivalis*, a gram-negative oral anaerobic bacterium known as an important pathogen in chronic periodontitis has been shown to respond to catecholamines released during stress processes by modifying their growth and virulence (Graziano et al. 2014). It occurs due to the increased expression of virulence and oxidative stress genes.

Adrenaline and noradrenaline can induce changes in gene expression related to oxidative stress and virulence factors. Experiments on gene expression show that production of L-DOPA and dopamine occurs in recombinant bacteria bearing the *Vitreoscilla* hemoglobin gene (Kurt et al. 2009). The activity of tyrosine phenol-lyase, the enzyme converting L-tyrosine to L-DOPA, was well-correlated to cytoplasmic L-DOPA levels.

Using a novel two-fluorophore chemotaxis assay, it was found that *E. coli* is attracted to epinephrine and norepinephrine (bacterial motility and biofilm formation of *E. coli* was also increased), while it is repelled by indole (Bansal et al. 2007). Moreover, epinephrine/norepinephrine upregulated the expression of genes involved in surface colonization and virulence, while exposure to indole decreased their expression (Bansal et al. 2007).

Catecholamines stimulate biomass accumulation (estimated nephelometrically) and cell proliferation (determined from an increase in colony-forming units) in non-pathogenic *E. coli* K-12 (strain MC4100) as shown by Anuchin et al. (2008). Dopamine stimulated proliferative activity and biomass accumulation to a greater extent than norepinephrine (Anuchin et al. 2008). Moreover, in prokaryotic and eukaryotic organisms present in humans, apart from cell proliferation, catecholamines stimulate the synthesis of adhesins, toxins, and other virulence factors (Lyte et al. 1996; Clarke et al. 2006). Biogenic amines, catecholamines, serotonin and histamine regulate the state and growth of yeast culture. Dopamine stimulated cell proliferation in the eukaryote *Saccharomyces cerevisiae*, while norepinephrine did not significantly influence this process (Malikina et al. 2010). Solutions of excretions containing biogenic amines constantly interact with bacteria that avoid high concentrations of the compounds by preventing exposure through the formation of biofilms (Oleskin et al. 2013).

It has been indicated that some microorganisms display resistance to catecholamines. Fungus *Cryptococcus neoformans* was unable to utilize catecholamines (epinephrine, norepinephrine, or dopamine) as a sole carbon or nitrogen source—which suggested that these compounds were not essential growth factors for that microorganism (Polacheck et al. 1990). The wild-type strain was found to be resistant to an oxidative system (epinephrine as an electron donor, Fe^{3+} as the catalytic transition metal ion, and hydrogen peroxide as an electron acceptor) due to phenoloxidase activity as a virulence factor, but mutants, which lacked the enzyme, were susceptible.

Serotonin. Serotonin stimulated cultural growth and cellular aggregation of bacterial species, including *Streptococcus faecalis*, the yeasts *Candida guilliermondii*, (Strakhovskaya et al. 1993) and *S. cerevisiae* (Malikina et al. 2010), *E. coli* K-12 and *Rhodospirillum rubrum* at concentrations of 2×10^{-7}–2×10^{-5} M (Oleskin et al. 1998a, b; Anuchin et al. 2008). Moreover, histamine showed similar effects on *E. coli* (Anuchin et al. 2007, 2008). Serotonin at 10^{-6}–10^{-5} M concentrations also inhibited light-dependent membrane potential generation in *Rsp. rubrum*, but in the myxobacterium *Polyangium* spp. serotonin stimulates cell aggregation and myxospore formation (Oleskin et al. 1998a, b). At concentrations near 20 μM, serotonin inhibits cell aggregation and microbial culture growth and photo-dependent membrane potential of the bacterium *Rsp. rubrum*. At micromolar amounts, the effects presumably result from the specific action of serotonin as an intercellular communication agent, accelerating, and possibly synchronizing, the development of the microbial cell population. According to Oleskin et al. (1998a, b), the growth stimulation of microorganisms by serotonin over a millimolar to micromolar range has been demonstrated in prokaryotes, both Gram-positive including *Streptococcus faecalis* (Strakhovskaya et al. 1993) and Gram-negative bacteria including *E. coli* and *Rsp. rubrum* (Oleskin et al. 1998a, b). In some cases, such as that for *Bacillus brevis*, the degree of growth stimulation achieved 100 % greater than that of a control.

Serotonin is thought to play an important role in the interactions between organisms in nature. The review by Oleskin et al. (2000) and monographs (Oleskin et al. 2010; Oleskin 2012) suggested that the integrity and coherence of microbial populations (colonies, biofilms, etc.) be viewed as unique so-called "superorganisms," which are thought to have become multicellular organisms over the course of evolution. This included such relevant phenomena as apoptosis, bacterial altruism, quorum effects, collective differentiation of microbial cells, and the formation of population-level structures such as an extracellular matrix. Emphasis can also be placed on the channels in colonies and agents of intercellular communication in microbial populations. Evolution has underscored the necessity of the involvement of a large number of evolutionarily-conserved communicational facilities and patterns of intercellular interactions. Moreover, an interesting fact is the 5-hydroxytryptophan conversion to serotonin under UV-irradiation (Fraikin et al. 1989). This neurotransmitter may serve as a protector for microorganisms in similar unfavorable conditions. For example, dinoflagellates (a large group of flagellate protists contained in marine plankton) and green algae *Gonyaulax polyedra* synthesize the protector melatonin, using serotonin as a precursor (Balzer et al. 1993). Circadian rhythms of indoleamines in the dinoflagellate *Gonyaulax polyedra* and persistence of the melatonin rhythm in constant darkness, have a relationship to 5-methoxytryptamine.

Histamine. Histamine synthesis by respiratory tract microorganisms was also observed, and its possible role in pathogenicity considered (Devalia et al. 1989). Much attention has also been given to the role of colony organization and intercellular communication in parasite/commensal/symbiont-multicellular host organism systems. Data from the literature on the ability of microorganisms to form plant hormones (biogenic amines) has been reviewed by Tsavkelova et al. (2006), who

discussed the *Rhodospirillum rubrum* pathways whereby the biogenic amines are metabolized, and their effects on the development and activity (physiological and biochemical) of the microorganisms are considered. The role as hormones and hormone-like substances is in the formation of association-type (microorganism-host) interactions. The histamine rise in wounds was a response of the victim to the snake containing the neurotransmitter in its venom (Lipps and Khan 2001), but bacterial histamine in this case may participate too (Lyte 2013).

2.3.1.2 Function in Plants

In plants, neurotransmitters demonstrate a high biological activity, playing a role as chemosignals, regulators of membrane permeability, growth and development regulators, etc. (Roshchina 1991, 2001a). Some examples will be considered below.

A signalling role of acetylcholine is seen in its participation in plant root-shoot signal transduction (Wang et al. 2003b; Baluska et al. 2004, 2005; Brenner et al. 2006). Acetylcholine causes rooting in leaf explants of in-vitro raised tomato (*Lycopersicon esculentum* Miller) seedlings (Bamel et al. 2007). Contractile effects of acetylcholine connected with membrane ion permeability were also observed in the regulation of the stomata function—the opening and closing movement in plants such as *Vicia faba* and *Pisum sativum* (Wang et al. 1998, 1999a, 2000). It was established that muscarinic and nicotinic acetylcholine receptors are involved in the event. A regulatory role for acetylcholine and its antagonists in inward rectified K^+ channels from guard cell protoplasts from leaf stomata of *Vicia faba* was found (Leng et al. 2000). Ca^{2+} and Ca^- ion-related systems were found to participate in acetylcholine-regulated signal transduction during stomata opening and closing (Wang et al. 2003a; Meng et al. 2004). A chloride channel in the tonoplast (vacuolar membrane) of *Chara corallina* also responds to acetylcholine (Gong and Bisson 2002). Electric processes participate in the electrical signaling, memory and rapid closure of the carnivorous plant *Dionaea muscipula* Ellis (Venus flytrap), and acetylcholine is thought to be involved in the phenomenon (Volkov et al. 2009). The formation of action potential in *Nitella flexilis* cells (Characeae) was studied, and acetylcholine (1–5 mM) activated potassium ion channels (Kisnieriene et al. 2012).

Acetylcholine and cholinergic systems play essential roles in plant fertilization and breeding. For example, lower activities of acetylcholinesterase and choline acetyltransferase in pistils (Tezuka et al. 2007) or in pollen (Kovaleva and Roshchina 1997) were associated with self-incompatibility. A role for acetylcholine can be proposed for its interaction with phytochrome and photoreceptor in growth regulation (Wisniewska and Tretyn 2003). There is a connection between some fungal infections (in particular for the *Fusarium* fungi) and the accumulation of plant growth regulators, gibberellic acid, and auxins. Acetylcholine and antibody against acetylcholinesterase may inhibit biosynthesis of gibberellic acid, one of the main growth hormones (Beri and Gupta 2007). The enzyme may also be included in

choline-auxin relations that affected plant growth processes. Direct evidence for the hydrolysis of choline-auxin or indole acetylcholine conjugates by pea cholinesterase has been demonstrated by some authors (Ballal et al. 1993; Bozso et al. 1995; Fluck et al. 2000). Germination of seeds, pollen, and vegetative microspores of various species was stimulated by acetylcholine as shown by many authors during the period of 1970–1992 (see references of Holm and Miller 1972; Tretyn et al. 1988 in monographs Roshchina 1991, 2001a). Acetylcholine growth-signal action was demonstrated in the auxin-dependent cell elongation in plants that may occur via vesicular transport (Di Sansebastiano et al. 2014).

Catecholamines may also serve as regulators of growth processes. For example, different concentrations of dopamine, noradrenaline and adrenaline stimulated the germination of seeds from *Raphanus sativus* (Roshchina 1991, 1992). Dopamine and noradrenaline also enhanced the same process in vegetative microspores from *Equisetum arvense* (Roshchina 2004). Unlike noradrenaline, dopamine acted in this manner only on the germination of pollen from *Hippeastrum hybridum* (Roshchina 2004). Catecholamines also stimulated the root-pumping activity via contractile proteins (Zholkevich et al. 2003, 2007a, b). This has been confirmed with the use of anticontractile compounds.

Dopamine is involved in the chemosignalling processes in plant microspores as well as in affecting contractile structures of plant cells (Roshchina 2005a, 2006a). High concentrations of dopamine (in the millimolar range) acting as strong oxidant-allelochemical decreased the root growth and cell viability in *Glycine max* (Guidotti et al. 2013). A defense function for catecholamines in the plant cell, related to their high amounts and ability to form toxic dopachrome, has also been considered in the literature (Roshchina 1991, 2001a; Szopa et al. 2001; Kulma and Szopa 2007). Increased dopamine content in some algae, in particular *Ulvaria obscura*, has led to the consideration of the neurotransmitter as a feeding deterrent (van Alstyne et al. 2006). This is a novel ecological role for a catecholamine. The confirmation of dopamine production acting as defense mechanism against grazers was done from experiments with isopods, snails, and sea urchin eating the agar-based foods contained exogenous dopamine. Damaged algae were also found to release a water-soluble reddish-black substance (dopachrome) that inhibits the development of brown algal embryos, reduced the rates of macroalgal and epiphyte growth and caused increased mortality in oyster larvae (Nelson et al. 2003).

Serotonin in many cases stimulated germination of seeds, vegetative microspores and pollen (Roshchina 1991, 1992, 2001a, b, 2004). This neurotransmitter plays a role in senescence in rice (Kang et al. 2009) and was shown to regulate root system architecture, perhaps, acting as an auxin inhibitor in *Arabidopsis thaliana* (Pelagio-Florez et al. 2011). There is a connection between indoleamine amines and serotonin biosynthesis in *Coffea canephora* P ex Fr. (Ramakrishna et al. 2012a, b). Serotonin (Roshchina 2001a) and its derivatives, such as melatonin (Posmyk and Janas 2009), also may play a protective role as antioxidants in various plants.

2.3.1.3 Functions in Animals

Currently, we have information about cellular functions for all animal organisms, including those which lack a nervous system and specialized functions unique to multicellular organisms that contain a nervous system. Neurochemical function is related to growth (similar between microbial and plant systems) and morphogenetic reactions. According to modern concepts, acetylcholine and serotonin may play a morphogenetic role in animals—from lower to higher animals (Buznikov 1990; Buznikov et al. 1996; Lauder and Schambra 1999).

The specialized function of neurochemical compounds concerned with the transmission of signals from one neuron to the next across synapses has been considered almost exclusively for neuronal systems as described in classical animal physiology. Neurotransmitters are also found at the axon endings of motor neurons, where they stimulate the contraction of muscle fibers. The first of the neurotransmitters to be studied, acetylcholine, transfers nerve impulses from one neuron to another, where it propagates nerve impulses in the receiving neuron, or from a neuron to a muscle cell, where it generates muscle contractions. Moreover, genetic defects of acetylcholine signalling promote protein degradation in muscle cells (Szewczyk et al. 2000). It is important to have proper nervous system and muscle function. In the adult nervous system, neurotransmitters mediate cellular communication within neuronal circuits.

Non-neuronal cholinergic system is found in different organs of mammalian organisms, in particular within blood lymphocytes and leukocytes where acetylcholine may play a role in the modulation of the immune system (Fujii and Kawashima 2001). Another group of authors (Pavlov et al. 2003) have suggested a link to the anti-inflammatory activity of macrophages via nicotinic acetylcholine receptors. In developing tissues and in primitive organisms, neurotransmitters influence growth and morphogenetic functions as regulators of embryogenesis (Buznikov 2007). They regulate growth, differentiation, and plasticity of developing central nervous system neurons. Cellular effects of acetylcholine in animals may also be related to pathogenesis of diseases such as acute and chronic inflammation, local and systemic infection, dementia, atherosclerosis, and finally cancer (Wessler et al. 2001).

Biogenic amines, dopamine, and serotonin act in worm *Caenorhabditis elegans* to modulate behavior in response to changing environmental cues (Suo and Ishiura 2013). These neurotransmitters act at both neurons and muscles to affect egg laying, pharyngeal pumping, locomotion and learning. A variety of experimental approaches including genetic, imaging, biochemical and pharmacological analyses have been used to identify the enzymes and cells that make and release the amines and the cells and receptors that bind them. Dopamine and serotonin act through receptors and downstream signaling mechanisms similar to those which operate in the mammalian brain suggesting that *C. elegans* will provide a valuable model for understanding biogenic amine signaling in the brain.

2.3.1.4 Possible Evolution of Neurotransmitter Reception

Since neurotransmitters are found in all living organisms—from unicellular to multicellular—Christophersen (1991) has described their possible evolution in terms of the molecular structure of neurotransmitters and adaptive variance in their metabolism, like that known for hormone receptors (Csaba 1980). The similarity of domains in signal receptors (Berman et al. 1991) was seen to compare with the physicochemical properties of signal receptor domains as the basis for sequence comparison. Christophersen (1991) advanced the hypothesis that all metabolites, even minor ones, are expressed as a result of stimuli and are directed against or support actions of receptor-based systems that reflect the evolution of receptors. For example, there is a similarity in some domains of rhodopsin, bacteriorhodopsin, and neurotransmitter receptors (Pertseva 1989, 1990a, b; Fryxell and Meyerowitz 1991). Recently, transgenic technique has permitted the expression of the human dopamine receptor in the potato *Solanum tuberosum* (Skirycz et al. 2005). A blockade of catecholamine-induced growth by adrenergic and dopaminergic receptor antagonists has been also observed for *E. coli* O157:H7, *S. enterica* and *Y. enterocolitica* (Freestone et al. 2007) The similarity and universality of basic endocrine mechanisms within the living world are shown in the examples of the development of receptor-based mechanisms of protozoa and invertebrates (Csaba and Muller 1996). There are conserved sections or domains in modern cholino- or aminoreceptors, which are also found in prokaryotes and had not changed over the course of evolution (Pertseva 1989, 1990a, b). Homology of some bacterial proteins (from *Mycobacterium smegmatis*, *Corynebacterium glutamicum*, and *Halobacterium salinarum*) to mammalian neurotransmitter transporters (for example, vesicular monoamine transporter) was also observed (Vardy et al. 2005). Today, molecular evolution of the nicotinic acetylcholine receptor has also been confirmed by the multigene family in excitable cells of highly organized animals (Le Novere and Changeux 1995). The nicotinic acetylcholine receptor (nAChR), a key player in neuronal communication, is thought to have been acquired by animals through early lateral gene transfer from bacteria (Changeux and Edelstein 2005).

Evolutionary questions are concerned with a recently identified bacterial adrenergic receptor which used eukaryotic signal molecules produced by the host that may have stimulated the virulence of gram-negative bacteria (Lesouhaitier et al. 2009). The sensitivity of prokaryotes to host signal molecules requires the presence of bacterial sensors (Grebe and Stock 1998). These prokaryotic sensors, or receptors, have a dual function: stereospecific recognition in a complex environment and transduction of the message in order to initiate bacterial physiological modifications. As messengers are generally unable to freely cross the bacterial membrane, they require either the presence of sensors anchored in the membrane or transporters allowing direct recognition inside the bacterial cytoplasm. The ability to recognize catecholamines may be related to the widespread utilization of catecholamines as signaling molecules in nature and the need for microbes to find a suitable host

(Freestone 2013), although a universal mechanism of bacterial recognition for animal and plant receptors has not yet been definitively identified. During evolution, microorganisms would have had to develop sensory systems which recognize biogenic amines as indicators. Recombinant systems include recombinant mouse brain serotonin receptor (5HT1c) to tobacco cells or oocytes *Xenopus laevis* (Beljelarskaya and Sutton 2003), and the receptor activity in such distinct organisms may confirm the evolutionary unity of reception in living communities.

2.3.2 Participation of Neurotransmitters in Chemical Communication Between Organisms

Secretion of neurotransmitters from any organism is the primary means by which a cell interacts with its surroundings, including animals, various plant species and microorganisms. Freestone (2013) discussed that a dialogue occurs between microbes and their hosts and that chemical signals are the language of this interkingdom communication. Through their long coexistence with animals and plants, microorganisms have evolved sensors for detecting eukaryotic hormones, which the microbe uses to determine that they are within proximity of a suitable host and to optimally time the expression of genes needed for host colonization. It has also been shown that some prokaryotic chemical communication signals are recognized by eukaryotes. Deciphering what is being said during the cross-talk between microbe and host is therefore important, as it could lead to new strategies for preventing or treating bacterial infections. Cross-relations between microorganisms living or parasitizing on plants and their possible virulence in human and economically-important animals via neurotransmitters and antineurotransmitter compounds, like ergot toxins, are significant as evidence in the field of microbial endocrinology. The chemical relations often are related to allelopathic interactions that take place in biocenosis with participation of compounds known as neurotransmitters (Guidotti et al. 2013; Roshchina and Yashin 2014). Mutual influence of organisms may be studied in models on which these interactions would be effectively explored. Cytodiagnostics of the cell-cell interactions is seen in the modeling of chemical relations where the secretions contain neurotransmitters and may react with other organisms (Roshchina 2014).

2.3.2.1 Microorganism–Microorganism Relations

Communication between microorganisms through their secretions (extracellular products) enriched in hormones or neuromediators has been proposed in many reports (Kaprelyants and Kell 1996; Kaprelyants et al. 1999; Oleskin et al. 2000; Kagarlitskii et al. 2003; Oleskin and Kirovskaya 2006; Oleskin 2007; Wang et al. 2013). Neurotransmitters participate in communication for growth; serotonin can act as an intercellular communication agent that may accelerate and, possibly,

synchronize the development of microbial cells. Exogenous serotonin stimulates the growth of yeast *Candida guilliermondii*, and the Gram-positive bacterium *Streptococcus faecalis* at low concentration near 10^{-7} M added at intervals of 2 h (Strakhovskaya et al. 1993). Photoactivation of the synthesis of endogenous serotonin in cells exposed to UV light at 280–360 nm led to the photostimulation of the same cultivated cells in lag-phase (Strakhovskaya et al. 1991; Belenikina et al. 1991). Exogenous serotonin at 2×10^{-7}–10^{-5} M also accelerates culture growth and induces cell aggregation in *E. coli* and *R. rubrum* (Oleskin et al. 1998a, b). Moreover, dopamine and norepinephrine stimulate the growth of *E. coli*, *S. enterica*, *Y. enterocolitica*, and the staphylococci as well as the yeast *Saccharomyces cerevisiae* (Neal et al. 2001; Kagarlitskii et al. 2003; Oleskin and Kirovskaya 2006; Freestone et al. 2007). Staleva et al. (2004) found that the catecholamines such as dopamine, adrenaline, and noradrenaline elevate some genes' (*FUS1* and *RLM1*) transcription in haploid *Saccharomyces cerevisiae* that is believed to have a possible interaction with bacterial cells via neurotransmitters released during periods of oxidative stress.

Interactions of different bacteria via catecholamines appear to occur as shown by Belay and Sonnenfeld (2002). Addition of culture supernatants from *E. coli* cultures that had been grown in the presence of norepinephrine was able to enhance the growth of *K. pneumoniae*. Addition of the culture supernatant fluid culture from *E. coli* cultures that had been grown in the presence of norepinephrine did not enhance growth of *P. aeruginosa* or *S. aureus*. Culture supernatant fluids from bacteria other than *E. coli* grown in the presence of norepinephrine were not able to enhance the growth of any bacteria tested. The results suggest that catecholamines can enhance growth of pathogenic bacteria, which may contribute to development of pathogenesis; however, there is no uniform effect of catecholamines on bacterial growth.

Recently a new model for the study of fungi-bacteria systems (to see the effect of added biogenic amines on the interaction between fungal hyphae on mycelium and bacterial biofilms) were presented by Oleskin and colleagues (2013) based on the fact that the organisms can synthesize norepinephrine and dopamine and release them into the culture fluid (Shishov et al. 2009; Oleskin et al. 2010; Oleskin 2012). Mycelium of the fungus *Coprinus comatus* being added into cultures of various bacteria such as *Bacillus subtilis*, *Arthrobacter globiformis*, *Pseudomonas fluorescens* may be covered by bacterial films. Upon contact, fungal-released serotonin, dopamine, or noradrenaline may inhibit the growth and proliferation of certain bacterial films (Oleskin et al. 2013).

2.3.2.2 Microorganism–Plant Relations

Activation of plant pattern-recognition receptors by microorganism occurs via a surface system. The first active layer of plant innate immunity relies on the recognition by surface receptors of molecules indicative of non-self or modified-self (Segonzac and Zipfel 2011). The activation of pattern-recognition receptors by pathogen-associated molecular patterns is in essence sufficient to stop pathogen invasion through transcriptional reprogramming and production of antimicrobials.

Communication between plant–microorganisms or plant–fungi via neurotransmitters is still a relatively unexplored field, although the presence of such compounds is documented for some fungi and rhizobial bacteria (Roshchina 2001a). The interactions between the pollen of plant *Haemanthus katherinae* (fam Liliaceae) and yeast *Saccharomyces cerevisiae* in natural conditions (without special addition of yeast in the at low to medium levels) or with the special addition of 1 mg/ml of the yeast are shown on Fig. 2.4. In the last image of Fig. 2.4 pollen tubes were absent, perhaps, due to a high concentration of catecholamines in yeast excretions.

Ishihara et al. (2008) experimentally found that the rice pathogenic infection by fungi *Bipolaris oryzae* (the formation of brown spots on the leaves) leads to enhanced serotonin production as a defensive response. In the defensive mechanism, the tryptophan pathway is involved as well. The pathway enzymes of rice have been characterized (Kang et al. 2007). Rice senescence induced by serotonin biosynthesis (Kang et al. 2009) may be a defense reaction against the pathogen. Participation of these neurotransmitters, as well as catecholamines, has been proposed in other areas of communication as well—between yeast *Saccharomyces cerevisiae* and in the leaves of fruit plants (Malikina et al. 2010).

2.3.2.3 Microorganism–Animal Relations

Microorganisms may live within an animal organism and have simple symbiotic or parasitic relationships with their host. An example of non-parasitic cooperation can be found in the marine sponge that uses acetylcholine and its hydrolyzing enzyme acetylcholinesterase of the associated bacterium *Arthrobacter ilicis* (Mohapatra and Bapujr 1998). The clinical aspect suggested by microorganism–animal interactions

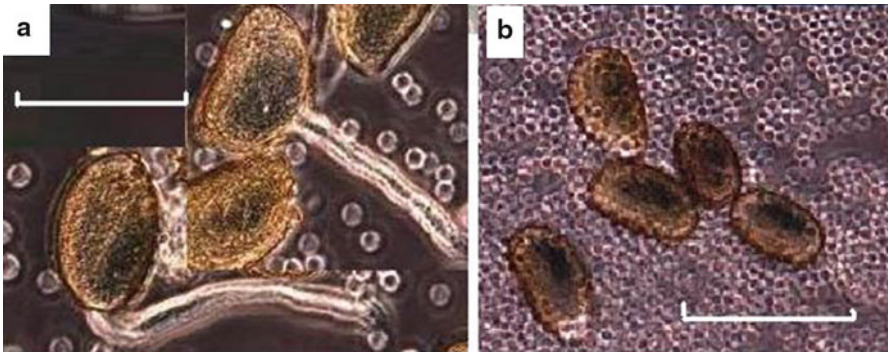

Fig. 2.4 The interaction between pollen of *Haemanthus katherinae* (fam. Liliaceae) and yeast *Saccharomyces cerevisiae* on the artificial medium—10 % sucrose (image seen under luminescence microscope Leica 6000 at excitation UV-light 405 nm + phase-contrast polarised light). Bars—50 μm. (**a**) Germinated pollen (pollen tubes seen) with small amount of surrounded yeast cells in natural conditions, (**b**) pollen with addition of 1 mg/ml of yeast in the medium, no pollen tubes in the variant

based on hormones is understandably of special interest. Evans and co-workers first reported in 1948 that catecholamines such as epinephrine were able to enhance bacterial infections. Presently, we know that they may stimulate the growth of Gram-negative bacteria (Lyte and Ernst 1992, 1993). The concept of "microbial endocrinology", in which pathogens are considered to exploit the host effector's molecules as environmental signals promoting growth and virulence factor deployment, has been proposed (Lyte 1992; Lyte and Ernst 1993; Freestone et al. 2008a, b). Cells of bacteria and fungi release neurotransmitters (for example norepinephrine and dopamine) out into the matrix of cellular cover as shown, in particular, in the bacterium *Bacillus subtilis*, where the compounds may participate in intercellular communication (Oleskin et al. 2000). Matrix contained biopolymers permit low-molecular neurotransmitters to diffuse among the colony population. In this case, the compounds serve as chemosignals or information agents which effect action within the radius of their short distribution area.

Lyte (1992) was the first to hypothesize and demonstrate that microbial recognition of catecholamines produced by the host during periods of stress were recognized and utilized by bacteria as a potential bacterial mechanism to initiate pathogenic processes (Lyte and Ernst 1993). Further, Lyte with coauthors (Lyte et al. 1996; Lyte and Bailey 1997; Freestone et al. 2008a, b) showed that norepinephrine stimulates the growth of low inocula of commensal and pathogenic *E. coli* in a minimal medium supplemented with serum. Norepinephrine also forms a complex with transferrin-bound iron in blood or serum, and Freestone et al. (1999, 2000) demonstrated that norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin. Utilization of iron-catecholamine complexes involving ferric reductase activity has also been found for *Listeria monocytogenes* (Coulanges et al. 1997).

Other examples are changes in the blood and tissue histamine content in rabbits when sensitized with streptococci combined with heart muscle extract (Kozlov 1972) as well in serotonin and histamine contents in organs infected with bacterium *Bacterium prodigious*. Host stress hormone norepinephrine stimulates *Streptococcus pneumoniae* pneumococcal growth, biofilm formation and virulence gene expression (Sandrini et al. 2014). The review by Freestone et al. (2008a) reveals that responsiveness to human stress neurohormones is widespread in the microbial world and relates to the new concept of microbial endocrinology. Mechanisms by which catecholamines induce growth in Gram-negative and Gram-positive human pathogens may include iron as an essential nutrient from host iron sequestering proteins (Freestone and Sandrini 2010). Bacteria in the gut, during early developmental stages of life, regulate brain levels of serotonin; colloquially known as the happy hormone (involved in the regulation of mood and emotion), serotonin is altered in times of stress, anxiety, and depression, and is a common active component in some of the most clinically effective antidepressant drugs (Clarke et al. 2012, 2013).

Today there is evidence of interaction between the gut microbiota and the brain that leads to regulation of neuronal development resulting in modifications of mental functions. Normal gut microbiota modulates brain development and behavior (Heijtz et al. 2011; Forsythe et al. 2012). According to Forsythe and Kunze (2013), the gut

bacteria have several effects on physiological processes such as communication between the intestines and the nervous system. Gut bacteria influence the development of the central nervous system (CNS) and stress responses. Application of *Lactobacillus rhamnosus* JB-1 or *Bacteroides fragilis* to carry the signal from microbe to neuron to the mucosal epithelium evokes sensory action potentials within a few seconds of administering the bacteria. Further understanding of the mechanisms underlying microbiome-gut-brain communication will provide us with new insight into the symbiotic relationship between gut microbiota and their mammalian hosts and help us identify the potential for microbial-based therapeutic strategies to aid in the treatment of mood disorders. Selection of a probiotic (good bacterium for the gut) is possible based on its neurochemical profile to specifically target host immunological processes (Lyte 2013).

Review by Freestone (2013) discussed that prokaryote responsiveness (in terms of enhancement of growth/virulence) to eukaryotic catecholamine hormones is widespread especially in bacteria inhabiting the gastrointestinal tract, particularly species such as *E. coli*, *Salmonella*, *Helicobacter*, *Listeria*, *Campylobacter*, and *Yersinia*. A distinct preference of each of these bacteria is found for the gut catecholamines noradrenaline and dopamine over adrenaline. It may be suggested that bacteria have evolved catecholamine response systems specific for the hormone they will encounter within their particular host niche.

Bacterial invasion could be the cause of ischemic disease, and catecholamines appear to prevent the bacteria-animal tissue association. Addition of physiologically relevant levels of norepinephrine induced dispersion of *Pseudomonas aeruginosa* biofilms that are associated with atherosclerotic carotid artery explants (Lanter et al. 2014).

Interactions like human platelet—bacterium *Staphylococcus aureus* 502A serve as an example of interaction that lead to release of serotonin and severe disorders including hemorrhage due to thrombocytopenia, disseminated intravascular coagulation. formation of septic emboli, occlusion of blood vessels and endothelial necrosis (Clawson et al. 1975). It is not excluded that serotonin may also be secreted from the bacterium.

2.3.2.4 Plant–Plant Relations

Within relationships between different plant species, neurotransmitters may play a role as an attractant or repellent for normal coexistence (Roshchina VV and Roshchina VD 1993; Roshchina 1991, 2001a). Plant microspores such as vegetative microspores of horse-tail *Equisetum arvense* from Cryptogam (spore-bearing) plants or various generative microspores (pollens) from Phanerogams (seed-bearing) plants are unicellular structures containing acetylcholine, catecholamines and histamine (Roshchina 2001a). And are specific microbiological items that have application and relevancy to medical areas of study (Roshchina 2006b), as they may act as drugs or allergenic agents.

An especially significant role of neurotransmitter compounds is seen in the pollen–pollen interaction named pollen allelopathy and pollen–pistil relations during pollination that regulate fertilization of certain plant species (Roshchina 2001b, 2007, 2008). Catecholamines, serotonin and histamine stimulate the germination of plant microspores (Roshchina 2001a, 2004, 2009) as well as dopamine regulate root growth (Guidotti et al. 2013). Fungi and other microorganisms living within a variety of plant cells also appear to release neurotransmitters that act as plant growth regulators. Currently, we may only speculate on the biological significance of these observations.

2.3.2.5 Plant–Animal Relations

Participation of neurotransmitters in plant–animal relations has been evidenced in dopamine (van Alstyne et al. 2006) and histamine (Swanson et al. 2004). In these pioneering studies, dopamine or histamine was released into a water medium by algae and affected the regulation of growth and development of the surrounding aquatic animals such echinoderms, mollusks, arthropods and sea urchins. This is dangerous for marine communities, fisheries and aquaculture facilities due to similar anti-herbivore defence that is the cause of reduced feeding by echinoderms, molluscs, and arthropods. To understand the mechanisms associated with dopamine release, van Alstyne and co-workers (2013) experimentally determined whether light quantity and quality, desiccation, temperature, exudates from conspecifics, and dissolved dopamine caused dopamine release (the concentration may be more than 500 μM). Although dopamine released into seawater can reduce the survival or growth of potential competitors, its release is associated with significant physiological stress and tissue mortality. As an anti-herbivore defense, the temperate green alga *Ulvaria obscura* demonstrates dopamine release and may serve as a sea model to study plant-animal interactions (van Alstyne et al. 2006, 2013, 2014). Red color of the algae population is concerned with the formation of a red oxidized product of dopamine called dopachrome. Van Alstyne with co-workers (2014) examined the effects of a range of dopamine concentrations on the growth of the green alga *Ulva lactuca*, on the germination of zygotes of the brown alga *Fucus distichus*, and on the survival time to metamorphosis and time to first molt of crab (*Metacarcinus magister* and *Cancer oregonensis*) larvae and juveniles . Dopamine began to inhibit *Fucus* germination at concentrations above 5 μM, *Ulva* growth at concentrations above 50 μM, and the survival of *Metacarcinus zoeae* at concentrations above 168 μM. It did not affect the survival of *Cancer megalopae* or juveniles or the time to metamorphosis of *megalopae*. It had no effect on the time to first molt of *Cancer* juveniles, except at the highest concentration tested (738 μM), where it delayed molting by an average of a day and a half. These toxic effects could have been due to the dopamine or to its oxidation products. Authors concluded that the large-scale release of dopamine by *U. obscura* following stressful environmental conditions could significantly affect co-occurring species in intertidal pools as well as intertidal and shallow subtidal marine communities where the alga can form large blooms.

Among other possible models of plant-animal relations in water larvae of the sea urchin *Holopneustes purpurascens* were suitable objects that underwent morphological change due to secretions from a host alga containing histamine (Swanson et al. 2004, 2007). This compound induced the settlement of larvae and that is why it may be a model to observe relations between plant and animal. Role of neurotransmitters excreted in the plant–animal relations is "terra incognita" as of yet. Although the phenomenon of allelopathy (includes all types of chemical relations between organisms in biocenosis), the occurrence of biogenic amines, such as the catecholamines and histamine in plant cells, has gained increasing interest within the scientific community (Roshchina and Yashin 2014).

2.3.2.6 Animal–Animal Relations

Neurosecretion utilizes mechanisms common to all eukaryotic modes of membrane transport, and the process should be a model of chemical secretion as a whole (Bajjalieh and Scheller 1995). The role of neurotransmitters affecting other organisms may be understood from the effects of the secretions released. For instance, large amounts of dopamine are secreted by cells of infusoria *Tetrahymena pyriformis* into their growth medium (Gundersen and Thompson 1985). Secretions from cones of *Drosophila* contain acetylcholine (Yao et al. 2000), and dopamine and norepinephrine are found in the salivary glands and brain of the tick *Boophilus microplus* (Megaw and Robertson 1974). Exogenous neurotransmitters serotonin and dopamine (10^{-7}–6×10^{-7} M) stimulate saliva secretion in insects (Marg et al. 2004) that have earlier been shown in model experiments on salivary glands of the cockroach *Periplaneta americana* (Just and Walz 1996). Microbial neurotransmitters may act by this mode. Moreover, the microflora enriched saliva found in insect bites are a potential source of human harm because they contain these neurotransmitters. Excreted dopamine and serotonin may act as both growth stimulators or as defence agents (Boucek and Alvarez 1970; Yamamoto et al. 1999).

2.3.2.7 Biomediator Role of Neurotransmitters

Non-neurotransmitter functions of the compounds known as neurotransmitters are especially important in the relationships of bacteria and fungi with plants and animals. Based on our present knowledge, one could imagine neurotransmitters rather as biomediators that, via cellular secretions, participate in cell–cell communications in biocenosis, i.e., a group of interacting organisms that live in a particular habitat and form a self-regulating ecological community (Fig. 2.5). Information about the intracellular location of the compounds and their release within a cell as well as their effects on the cellular organelles could be useful in the study of cellular endocrinology. The role of acetylcholine and biogenic amines as intracellular regulators has been confirmed for sea animals (Buznikov 1990; Buznikov et al. 1996) and for

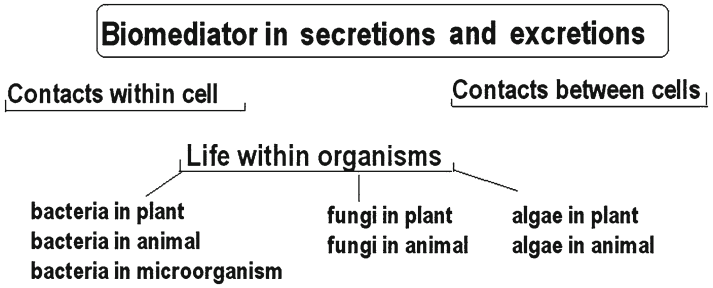

Fig. 2.5 Possible relationships which involve biomediators

some plants (Roshchina 1989, 1990a, b, 1991, 2006a, b). A special case is related to the life of microorganisms within the host cell of the animal or plant. The release of neurotransmitters should occur within and outside the host cell (parasitic or otherwise). A universal (biomediator) role of neurotransmitters may be a subject of future investigations.

2.4 Use of Microorganisms and Medicinal Plants Enriched in Neurotransmitters and Anti-neurotransmitter Compounds

Special interest for pharmacology and medicine is in the search of natural excretions from plant and microorganisms that may hold value as a potential drug candidate. The original materials for the drugs may be both microorganisms and plants. For example, anticholinesterase compounds may be used in the treatment of glaucoma, myasthenia, Alzheimer's disease and other diseases when there arises a need to support a normal concentration of acetylcholine in the blood and brain. Among such substances are inhibitors of cholinesterases from fungi, such in mushrooms. Today about 260 of these classified compounds have been isolated from plants (Filho et al. 2006). The isolated substances are mainly alkaloids that prevent formation of amyloid plaques and are derived from quinolizidine, steroidal, indole, and isoquinoline structures (Dall'Acqua 2013)

2.4.1 Microbial Neurotransmitters

Stress diagnostics. Stress stimulates the formation and release of biogenic amines, including epinephrine, a hormone produced during stress that affects heart rate, blood circulation and other functions of the body. Microorganisms possess the ability to recognize hormones within the host and utilize them to adapt to their

surroundings. Norepinephrine and epinephrine, which are released during human stress responses, may act as environmental cues to alter the growth of individual microbes. Growth can be stimulated in over 42 oral bacteria by norepinephrine and epinephrine (Roberts et al. 2002), including *Actinomyces naeslundii*, *A. gerencseriae*, *Eikenella corrodens*, and *Campylobacter gracilis*. This suggest that stress induced change in local catecholamine levels in the mouth may play a significant role in the etiology and pathogenesis of periodontal disease. Increased concentration of these compounds due to a bacterial presence may be a valuable diagnostic test in medical practice.

Bioassay for drug testing. It may be possible to use bacterial chemotaxis as a bioassay in biochemical studies of drug action because the bacterium *Pseudomonas fluorescens* is sensitive to catecholamines and acetylcholine (Chet et al. 1973)

Tools for chemistry. Chemical reactions performed by microorganisms have been used as a modern tool in chemistry. In the work of Boaventura et al. (2004), the ability of the fungi *Beauveria bassiana* and *Aspergillus niger* to modify the chemical structure of indole compounds was studied. *B. bassiana* was able to transform 3-indolylacetonitrile into 3-methylindole, while *A. niger* transformed tryptamine into 5-hydroxyindole-3-acetamide. These fungi were able to perform both the reduction and oxidation of the indole compounds, the oxidation occurred with improvements in oxygen uptake. The synthetic use of microorganisms to perform reactions in the indole nucleus of serotonin is of industrial interest because the bacterium can synthesize active indole derivatives and so has attracted great attention. According to Heller et al. (2004), serotonin also enhances the activity of the membrane-acting drug amphotericin B against *Aspergillus fumigatus* in-vitro. Thus, the combination of known drugs with biogenic amines may allow for the creation of novel medical applications. Since neurotransmitters may be released from organisms and influence other organisms (both of the same species or different ones), a new focus in such studies should include analysis of the interaction of mechanisms which are related to the neurotransmitter compounds and anti-neurotransmitter compounds in the secretions. Of special interest are aquatic organisms (Pandey et al. 2014). The majority of the active isolates were colonizing bacteria of soft corals followed by sediment isolates while most of the potent inhibitors belonged to the colonizing bacteria of marine sponges. For example, marine sponge *Fasciospongia cavernosa* associated strain of *Bacillus subtilis* and other marine bacteria have been shown to produce anticholinesterase compounds which inhibit the electric eel enzyme, acetylcholinesterase (Pandey et al. 2014). Their activity has been compared to galanthamine and it may be supposed that the marine source (an easily obtainable natural source) was applied for the mass production of these therapeutic compounds. From this perspective, it should be kept in mind that similar inhibitors reduce the activity of enzyme acetylcholinesterase that degrades the neurotransmitter acetylcholine in the brain and leads to retardation in neurodegenerative diseases like Alzheimer's, Parkinson's, and other diseases. A marine sponge associated strain of *Bacillus subtilis* and other marine bacteria can produce anticholinesterase compounds that also may be used in medicine (Pandey et al. 2014). Although, plants

that possess such substances have also been a significant source of these compounds. Some of the compounds may have therapeutic significance.

Probiotics. In the last 100 or so years, several drugs and biosupplements called probiotics have gained significance. Among the organisms that benefit health when consumed are bacteria and yeasts (Rijkers et al. 2011). The most well known, *Bifidobacterium longum* has found great application in the gut. Neurotransmitters derived from these microorganisms, such as probiotics, appear to be compounds that may have a role in health and disease (Lyte 2013). Lyte (2011) hypothesized that the ability of probiotics to synthesize neuroactive compounds provides a unifying microbial endocrinology-based mechanism to explain the hitherto incompletely understood action of commensal microbiota that affect the host's gastrointestinal and psychological health. Once ingested, probiotics enter an interactive environment encompassing microbiological, immunological, and neurophysiological components.

2.4.2 Plant Neurotransmitters

Plants may be suitable for medicinal applications as a source of neurotransmitters and neurotransmitter-based drugs or as a platform for testing of the neurotransmitters and neurotransmitter-based drug compounds. Further, plants may serve as a model system for use in examining cellular endocrinology-based mechanisms. It should be pointed out that pharmacologically valuable plants can serve as a valuable source material for neurotransmitters. Examples of plants that serve as a source of neurotransmitters have been previously documented in the literature (Roshchina 2001a). Among earlier published studies are recommendations for medical applications of acetylcholine-enriched food, for example *Digitalis ferruginea* and *Urtica dioica*, catecholamine-enriched *Musa* sp. (Roshchina 2001a), serotonin-enriched *Hippophae rhamnoides*, *Juglans nigra*, and *J. regia* (Bell and Jansen 1971; Badria 2002). Useful features of medicinal plants enriched in neurotransmitters are likely connected with the formation of non-toxic complexes with neurotransmitters such as conjugates of auxins (Ballal et al. 1993; Bozso et al. 1995; Fluck et al. 2000) or phenol–histamine (Hikino et al. 1983). Possession of acetylcholine receptor binding activity is unique to many medicinal plants used to improve failing human memory (Wake et al. 2000; Luedtke et al. 2003). Natural anti-neurotransmitter compounds may also be used in medicine. Agonists or antagonists of neurotransmitters as well as anticholinesterase compounds, mainly alkaloids (Schmeller et al. 1997) or terpenoids (Atta-ur-Rahman et al. 2001), occur in pharmacologically valuable plant material and are effective against a range of new and ancient diseases (Roshchina 2001a). For example, the alkaloids berberine, palmitine, and sanguinarine (inhibitors of cholinesterases, choline acetyltransferase or other receptors) are toxic to insects and vertebrates and inhibit the multiplication of bacteria, fungi, and viruses (Schmeller et al. 1997).

Model systems of plants may also be used for drug testing (Roshchina 2014). New approaches to the testing of neurotransmitter and anti-neurotransmitter compounds may be in the plant biosensors found among sensitive microobjects, in particular plant microspores such as vegetative horse tail microspores or the generative male microspores called pollen (Roshchina 2004, 2006a, b, 2007). Biosensors are analytical systems, which contain sensitive biological elements and detectors. Intact plant cells are a possible biosensor, having a natural structure that determines their high activity and stability (Roshchina 2006a, b; Budantsev and Roshchina 2004, 2007). Changes in the germination and autofluorescence of unicellular microspores of plants as well as their cholinesterase activity were considered potential biosensor reactions (Roshchina 2005a, b). They could serve as biosensors for medicinal drugs such as known agonists and antagonists of neurotransmitters, replacing the use of vivisectioned animals.

Plants appear to be model systems for cell endocrinology. Unicellular plant systems such as the above-mentioned microspores are also suitable models for cellular endocrinology studied within unicellular and multicellular organisms. This allows for a better understanding of neurotransmitter presence in organelles and different compartments which is based on the concept of universal mechanisms in intracellular chemical signalling from plasmalemma to organelles (Buznikov 1990; Roshchina 1989, 1990a, 2001a; Roshchina et al. 2015b).

The study of neurotransmitter function and location within a cell could be performed with fluorescent compounds from microbial and plant cells that bind with the receptors or enzymes of neurotransmitter metabolism (Roshchina 2008). For example, the fluorescent anti-neurotransmitters *d*-tubocurarine, muscarine (Roshchina 2005a, b, 2008), and some Bodipy derivatives of neurotransmitters (Roshchina et al. 2003; Roshchina 2008) are used as fluorescent natural dyes and markers because they bind with cellular receptors.

2.5 Future Perspectives and Considerations

Approximate analysis of the evolution of non-neuronal functions of neurotransmitters in various kingdoms reveals the paucity of data at the present time:

1. Identification of acetylcholine (choline acetyltransferase and cholinesterase) and biogenic amines (enzymes of their metabolism) in microorganisms and plants as well as their metabolic pathways have both similarities and differences to mammalian metabolism.
2. Studies characterizing cellular reactions, including electric, growth, and motile responses, and whether these reactions are common or distinct between animals—either Vertebrata or Invertebrata.
3. Identification of new models (like *E. coli*, drosophila, maize, rice, Arabidopsis that served as wide-known models in genetics) suitable for the observation of cellular reactions.

4. Inhibitory (pharmacological) analysis of the receptors presence and possible genetic identification in non-neuronal systems.
5. Analysis of the participation of neurotransmitters (biomediators) in symbiotic or parasitic relations of microorganisms within plant or animal organisms.
6. Investigation of ecological and medicinal importance of microbial endocrinology resulted in the creation of necessary methodologies and manuals for the control of neurotransmitters in food, water and air.
7. The application of the above-mentioned trends in understanding the universality of the chemical nature of communication and response-reaction based on the presence of neurotransmitters (biomediators) in any living cell.

In the future, all above-mentioned investigations may help to create a common map that relates and clarifies the function of neurotransmitters as well as their development over the course of evolution. What is a neurotransmitter's original role—either evolutionarily-speaking or perhaps fundamentally within an organism? We must consider extracellular events related to the compounds—their release and connection with sensitive cells (primitive chemotaxis). Modern approaches to evolutionary investigations may include mammalian or plant enzymes involved in the synthesis or catabolism of neuromediators in bacterial cells. This may help determine similarities or distinctions in an enzyme's activity and the degree of divergence of the enzyme from another during the process of evolution. Special attention should also be given to the involvement of neurotransmitter-biomediators in intracellular signalling—that is, from one organelle to another or from one compartment to another because the use of neurotransmitter-biomediators in communication can be a primary function of biomediators in bacterial cells.

2.6 Conclusion

Examination of the compounds known as neurotransmitters or biomediators reveals a similarity in their main functions at the cellular level for all living organisms. These compounds change the membrane ion permeability and electrical characteristics of the cells and, overall, we see the response of the cell or organism as stimulation or inhibition of its growth and development. Neurotransmitters regulate their own metabolic processes within a cell and the relationships (allelopathy) between neighbors with biocenosis. And, these molecules may serve as attractants or repellents as well as oxidative agents (biogenic amines). Interactions between microorganism–microorganism, microorganism–animal (human) and microorganism–plant play essential roles in the environment, and thus neurotransmitter compounds should be considered universal agents of communication that are instrumental in determining reaction-response interactions in existing relationships. We must understand that our current knowledge of these relationships is limited and that the field is still in its infancy. However, increasing recognition and understanding of such relationships is rapidly changing medical and pharmacological perspectives of the non-nervous system functions of neurotransmitters.

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Chapter 3
Catecholamine-Directed Epithelial Cell Interactions with Bacteria in the Intestinal Mucosa

David R. Brown

Abstract The catecholamines epinephrine, norepinephrine and dopamine are present in or have access to mucous membranes in the digestive, respiratory and genitourinary tracts, which represent the first sites of microbial colonization and infection within the body. Epithelial cells at mucosal surfaces establish and maintain symbiotic microbial communities and serve as the initial cellular point of contact for pathogens with the animal host. These cells express receptors that are capable of detecting and responding to microbe-associated molecular patterns and in most host species express G protein-coupled receptors for catecholamines. Although it is increasingly recognized that substances produced and released from nerves and endocrine cells can exert immuno-modulatory actions at mucosal sites, there have been few investigations focused specifically on the catecholaminergic modulation of interactions between the mucosal epithelium and bacteria or other mucosa-associated microorganisms. The potential biomedical importance of this phenomenon cannot be understated. For example, psychological stress or other conditions that activate the sympathetic nervous system to release epinephrine and norepinephrine may act to produce short-term changes in luminal and mucosal microbial communities or alter the course of a bacterial infection. This chapter will briefly review this developing and important research area of mucosa-microbe interactions with a focus on intestinal host defense.

Abbreviations

AR	Adrenergic receptor
DA	Dopamine
DAR	Dopamine receptor

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EHEC	Enterohemorrhagic *Escherichia coli*
EPI	Epinephrine
MAMP	Microbe-associated molecular pattern
NE	Norepinephrine
pIgR	Polymeric immunoglobulin receptor
SIgA	Secretory immunoglobulin A

3.1 Introduction

The mucosal surfaces that line body cavities, such as those in the gut, airways and female reproductive tract, are common sites for localized microbial infections and serve a vital role as highly effective barriers against systemic infection. There is mounting evidence that mucosal barrier function and other anti-microbial defense mechanisms are developed and sustained in part by diverse and regionally-specific populations of commensal microflora that extensively colonize mucosal surfaces at different body microenvironments. Although mucosal surfaces have been known for decades to harbor resident microflora, the existence and biomedical importance of complex, microbial endosymbiont communities to the health and well-being of the host—a role encapsulated in the "microbiome" concept—has only recently captured the imaginations of scientists and the lay public alike (Savage et al. 1968; Redinbo 2014). Although the resident microflora were initially circumscribed to viable and culturable bacteria, it is now apparent that mucosal microbial communities represent not only previously unknown bacterial species, but a diverse array of mucosa-associated viruses, fungi, and parasites as well (LaTuga et al. 2011).

Epithelial cells represent the predominant cellular component of mucosal tissues and are usually the first cell type to come into contact with microbes at mucosal surfaces. Depending upon their location and characteristics of the microbial communities they support, these cells are capable of recognizing specific microbial-associated molecular patterns (MAMPs) and orchestrating immune responses to them (Hirota and Knight 2012; Patten and Collett 2013). In addition, mucosal epithelial cells express a variety of membrane-associated ionotropic and metabotropic receptors for extracellular neuromodulatory substances and hormones that have traditionally been thought to function solely in the modulation of transepithelial ion transport and barrier function. At this point in time however, it is has become clear that these host cell receptors additionally regulate other roles of mucosal epithelial cells, including interactions between this host cell type with microbes and microbial products.

In this review, the known and potential actions of catecholamines (epinephrine, norepinephrine and dopamine) in modulating interactions between epithelial cells and bacteria at the mucosal surfaces of the intestine, will be discussed. In most cases, catecholamine effects on epithelial cells are mediated by pharmaco-

logically (i.e. functionally)-defined adrenergic receptors (ARs) and, in some cases, biochemically-defined specific binding sites for catecholamines on epithelial cell membranes. These highly-regulated receptors are coupled to guanine nucleotide-binding (G) proteins which, when activated, modulate levels of intracellular cyclic nucleotides and other signaling molecules that are linked to epithelial function.

The apparent inter-kingdom communication between prokaryotic bacteria and eukaryotic host epithelial cells at mucosal surfaces is a thought-provoking concept encapsulated in the term, "microbial endocrinology". Signaling between these taxonomically different types of cells involves a wide variety of extracellular molecules that are generated and secreted from one cell type and produce biological effects in target cells. Catecholamines, and more recently opioids (Zaborina et al. 2007; Babrowski et al. 2012; Zaborin et al. 2012), that are synthesized in eukaryotic cells have been found to alter the growth and virulence properties of bacteria. Epinephrine (EPI) and norepinephrine (NE) have also been shown to increase the infectivity of an enteric parasite (Coppi et al. 2002). This is an important subject discussed elsewhere throughout this book, and it will not be further considered here. A role for adrenergic receptors (which have been defined traditionally through experiments in animals or animal tissues) in mediating the direct actions of catecholamines on bacteria remains to be established by pharmacological criteria. It is not necessary to invoke an adrenergic receptor-mediated mechanism of action in bacteria for these substances, however. Indeed, a recent study indicated that *E. coli* chemotaxis in response to NE is due to NE conversion to 3,4-dihydroxymandelic acid, a NE metabolite which interacts with a bacterial serine chemoreceptor that mediates bacterial movement (Pasupuleti et al. 2014). Moreover, the catechol moiety present in catecholamines is also present in some animal and bacterial siderophores, e.g. siderocalin (Sia et al. 2013) and salmochelin (Müller et al. 2009). Catechol groups have the ability to bind to ferric iron and this property may underlie the direct effects of NE or EPI on bacterial function, as has been reported for *Helicobacter pylori* and *Streptococcus pneumoniae* (Doherty et al. 2009; Sandrini et al. 2014). Indeed, iron transfer by catecholate molecules may contribute to the establishment of the gut microbial community (Pi et al. 2012).

3.2 Locations of Catecholamines and Catecholamine Receptors at Mucosal Sites

Peripheral catecholamines within mammalian hosts are produced by and released from four sources (Kvetnansky et al. 2013): sympathetic nerves, the adrenal gland, autocrine-paracrine systems involving dopamine (Goldstein et al. 1995), and certain types of non-neuronal cells. In all cases, these catecholamine-synthesizing cells express tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis. The predominant catecholamine stored in synaptic vesicles and released

from nerves innervating the intestines and many other regions encompassing the common mucosal immune system is NE. Dopamine (DA), the immediate precursor of NE, is often co-released albeit to a relatively lesser extent. Peripheral nerves do not have the ability to form EPI because they lack the enzyme phenylethanolamine *N*-methyl transferase, which catalyzes the *N*-methylation of NE to EPI. This enzyme is abundantly expressed in chromaffin cells of the adrenal medulla, which represent the main source of circulating EPI. In addition to releasing EPI and NE, adrenochromaffin cells have been shown to secrete peptides possessing antimicrobial activity, including some derived from the endogenous opioid peptide precursor molecule, pro-enkephalin A (Metz-Boutigue et al. 1998).

3.2.1 Catecholamines in the Intestinal Mucosa

Both DA and NE are synthesized in and released from secretomotor nerves that project to the intestinal mucosa. These nerves display immunoreactivity for tyrosine hydroxylase, and those capable of producing NE additionally express immunoreactivity for dopamine beta-hydroxylase. The noradrenergic innervation of the intestine originates in neurons lying outside the gut wall located in prevertebral ganglia, but dopaminergic neurons appear to reside within the intestinal wall (Anlauf et al. 2003; Li et al. 2004; Furness 2006). Noradrenergic nerves that regulate submucosal blood flow do so through direct interactions with arterioles, whereas adrenergic nerves affecting epithelial function can do so indirectly by modulating the activity of neurochemically-different classes of secretomotor neurons innervating the mucosa, at least in some animal species or intestinal segments (Brown and O'Grady 1997; Furness 2006). Subsets of noradrenergic nerves co-express (and co-release) other neuromodulatory substances, such as neuropeptide Y (Furness 2006). Similar to some adrenochromaffin cell peptides discussed above, neuropeptide Y and some other classes of neuropeptides have been found to possess antimicrobial activity (Brogden et al. 2005).

In addition to neuronal cells, immune cells appear to be capable of synthesizing, releasing, and degrading catecholamines. There is an abundance of immunocytes throughout the intestinal lamina propria, and these cells, including mast cells in some animal species, may represent an alternate, non-neuronal sources of DA and/or NE in the intestinal wall (Falck et al. 1964; Vieira-Coelho and Soares-da-Silva 1993; Flierl et al. 2008). In mice, free NE and DA released from enteric neural and non-neural cells can be detected in the luminal fluid of the intestine (Asano et al. 2012). It is likely that the sources of these luminal catecholamines are non-neuronal cells as well as enteric nerve endings.

The Peyer's patch is a major inductive site for mucosal immunity in the intestinal tract. Peyer's patches are organized lymphoid follicles covered by a single layer of specialized epithelial cells (i.e. M cells) that sample gut luminal contents for potentially harmful microbes and antigens and subsequently initiate adaptive immune responses, leading to the generation of dimeric immunoglobulin A (IgA)-producing

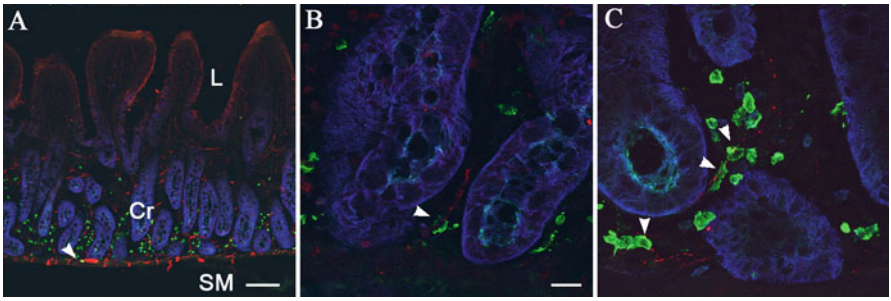

Fig. 3.1 Proximity of enteric cholinergic and adrenergic nerve fibers to IgA-expressing plasma cells and secretory component (SC)-expressing epithelial cells in porcine ileum. Confocal micrographs are representative of observations in a minimum of three transverse tissue sections from each of five pigs. (**a**) Nerve fibers immunoreactive for the neural marker PGP 9.5 (*red*) are in close proximity to both IgA-immunoreactive plasma cells (*green*) and epithelial cells immunoreactive for SC (*blue*) in the ileal crypts. (**b**, **c**) Higher magnification shows nerve fibers (*arrowheads*) immunoreactive for the vesicular acetylcholine transporter (*red*, **b**) and dopamine beta-hydroxylase (*red*, **c**) closely apposed to B lymphocytes immunoreactive for IgA (*green*) and epithelial cells immunoreactive for SC (*blue*). *Cr* ileal crypt, *L* intestinal lumen, *SM* submucosa. Scale bars: (**a**) 100 μm; (**b**, **c**), 15 μm (L.D. Schmidt, L. Vulchanova and D.R. Brown, unpublished data)

lymphoblasts (Brandtzaeg 2013). There is accumulating evidence from several animal species that Peyer's patches are innervated. In the porcine and ovine small intestine, for example, there is strong immunohistochemical evidence for catecholaminergic innervation of Peyer's patches (Kulkarni-Narla et al. 1999; Green et al. 2003; Chiocchetti et al. 2008). Tyrosine hydroxylase- and dopamine beta-hydroxylase-like immunoreactive nerves terminate beneath Peyer's patch epithelia. Moreover, enteric nerves terminating near Peyer's patch follicles in swine express immunoreactivities for the type 2 vesicular monoamine transporter (SLC18A2), which transports cytosolic catecholamines into synaptic vesicles, and the sodium-dependent NE transporter (SLC6A2), which takes up extracellular NE into presynaptic nerve terminals (Kulkarni-Narla et al. 1999; Green et al. 2003).

Lymphoblasts committed to dimeric IgA production mature in the systemic circulation and subsequently traffic to effector sites distributed diffusely throughout the intestinal mucosa. Indeed, a majority of the mucosal tissue supports immune effector function through the vectorial, transcellular secretion of secretory IgA (SIgA; see below) from B lymphocytes. In swine, both cholinergic and noradrenergic nerve fibers terminate in close proximity to lymphocytes and epithelial cells that are immunoreactive for IgA and secretory component respectively in the crypt regions of the small and large intestinal mucosae (Fig. 3.1; Schmidt et al. 2007; L.D. Schmidt, L. Vulchanova and D.R. Brown, unpublished observations).

3.2.2 A Few Words About Catecholamine Receptors

Norepinephrine, EPI and DA activate their cognate G protein-coupled receptors expressed on neurons, epithelial cells and other types of target cells which influence overall mucosal function and alter intestinal susceptibility to infection. Receptors for NE and DA have been defined over several decades through the use of highly selective receptor agonists and antagonists in functional pharmacological investigations and more recently, through molecular cloning and structure-function studies in isolated cells and transgenic animals.

The receptor concept was developed in the early twentieth century on the translational approach of defining binding interactions of endogenous substances or their synthetic homologs with specific sites ("receptive substances") expressed by target cells that are linked to a biological response (Rang 2006). Biochemical analyses of selective binding site interactions with catecholamine-like drugs and other ligands that were developed many years later have provided crucial information on the affinities (K_d, dissociation constant) and competitive interactions of catecholamines and their synthetic derivatives at specific binding sites. However, these biochemical studies do not measure the *biological activity* of the ligands examined, and therefore they do not truly define a "receptor", which is an entity functionally coupled to intracellular signal transduction pathways that mediates a biological function. In other words, biochemical analyses of binding sites cannot distinguish between agonist and antagonist ligands. Binding studies, if carefully executed, can provide information on a specific, high-affinity binding site for an endogenous or synthetic ligand that can serve as supporting evidence for the presence of a true receptor in a biological system. Through GTPγ^{35}S binding assays (Harrison and Traynor 2003), it is possible to assess the effectiveness of ligands to activate G proteins coupled to a particular receptor and this approach affords a better approximation of ligand activity at the cellular level (e.g. it is possible to distinguish an agonist from an antagonist).

Functional criteria for a receptor-mediated effect include (1) selective antagonism, (2) a rank order of ligand potency (usually based on the 50 % effective concentration, EC_{50} for agonists or a pK_B value for antagonists, which is determined through rightward shifts in an agonist concentration-effect relationship after pretreatment with a competitive antagonist) that is consistent with the pharmacological signature of a particular receptor type; and (3) stereospecificity (e.g. the levorotatory isomer of NE is much more potent than its dextrorotatory counterpart at ARs). The affinities and competitive interactions among ligands for ARs and dopamine receptors (DARs) measured by functional drug interactions are generally well correlated with the affinities and interactions of the same ligands determined biochemically at specific binding sites.

Presently, ARs are classified into alpha- and beta-AR types; this includes two subtypes of *alpha*-ARs (*alpha*$_1$ and *alpha*$_2$) having three isoforms each, and three subtypes of *beta*-ARs. An EPI-preferring *beta*$_2$-AR was the first drug receptor to be cloned and characterized in the modern era of molecular pharmacology (Dixon et al. 1986). Compared to EPI, NE has relatively higher binding affinity for *alpha-ARs* and *beta*$_1$- and *beta*$_3$-ARs, but a lower affinity for *beta*$_2$-ARs. Two main DAR types

exist through gene duplication events in the vertebrate lineage (DA_1R and DA_2R); from these, five receptor subtypes have been defined. Our understanding of the nature of these receptors and their relationships to G proteins and downstream intracellular signaling cascades continues to grow. For example, there is accumulating evidence that ARs may form dimeric complexes on cell membranes that possess a pharmacological profile different from that of the monomeric receptor (Verburg-van Kemenade et al. 2013). Moreover, *beta*-ARs can signal differentially through intracellular G proteins and *beta*-arrestin, and thereby produce different ligand-dependent biological effects (Bock et al. 2014). This should be kept in mind when designing experiments involving the prolonged contact of catecholamine agonists with their receptors, as long-term stimulation of the receptor can often result in its phosphorylation, which in turn can increase interactions of the receptor with arrestin or other intracellular proteins associated with receptor down-regulation. The investigator is then left with the question of whether the biological effects examined were the result of receptor activation or the loss of receptors from cell membranes.

3.2.3 *Localization of Catecholamine Receptors on Mucosal Epithelial Cells*

As indicated above, pharmacologically-defined catecholamine receptors influencing epithelial function may be expressed on epithelial cells or on nerves innervating these cells. Specific, high-affinity binding sites for catecholamines have been detected on mucosal epithelial cells in some species, including the guinea pig and rat. These include $alpha_1$-, $alpha_2$- or *beta*-adrenergic binding sites as well as DA and non-adrenergic imidazoline binding sites (Chang et al. 1983; Nakaki et al. 1983; Cotterell et al. 1984; Paris et al. 1990; Senard et al. 1990; Valet et al. 1993; Baglole et al. 2005). Food deprivation appears to increase $alpha_2$-AR binding sites on rat jejunal epithelial cells (Lucas-Teixeira et al. 2000) and pro-inflammatory cytokines and short-chain fatty acids generated by colonic bacteria may decrease the rate of $alpha_2$-AR gene transcription and hence, the number of binding sites in human HT-29 colonic epithelial cells (Devedjian et al. 1996; Cayla et al. 2008).

In other species, *alpha*-ARs modulating epithelial functions appear to be expressed solely on nerve membranes, at least in some regions of the intestinal tract. Synaptosomal preparations enriched in ^{3}H-saxitoxin binding sites were obtained from the submucous neural plexuses of the canine and porcine small intestine. In these neural preparations, saturation analyses indicated that highly-selective *alpha2-AR* antagonists bound specifically to these enteric neural membranes with a dissociation constant (K_D; a measure of ligand affinity) of 3.5 nM [^{3}H-rauwolscine, dog; Ahmad et al. 1989] and 0.4 nM, respectively [^{3}H-yohimbine, pig; Hildebrand et al. 1993]. In a study with porcine small intestine, specific binding of ^{3}H-yohimbine to intestinal epithelial cells was minimal (Hildebrand et al. 1993). On the other hand, in the porcine colonic mucosa, the functional effects of catecholamines on electrolyte transport are mediated by $alpha_1$-ARs and are resistant to the neural conduction

blocker tetrodotoxin, indicating that these substances may act on epithelial cell receptors in this gut segment (Traynor et al. 1991). Whether or not specific *alpha1-AR* binding sites exist on colonic epithelial cell membranes in pigs has not been determined.

3.3 Catecholamines and Mucosal Interactions with Bacteria

Both NE and DA have been shown to alter the mucosal attachment or invasiveness of bacterial pathogens such as enterohemorrhagic *Escherichia coli* (EHEC) or serovars of *Salmonella enterica* by acting on the intestinal mucosa. The actions of these catecholamines on bacteria-mucosa interactions have been examined in mucosal explants from murine or porcine intestine mounted in Ussing chambers (Brown and O'Grady 2008). This apparatus has been used for decades in studies of transepithelial ion transport and more recently, in investigations of bacteria on intestinal transport (Lomasney and Hyland 2013). The Ussing chamber system extends the viability of mucosal explants under quasi-physiological conditions, allows for tangential flow of bacteria across a fixed mucosal surface area, and permits the selective contact of drugs and bacteria with the luminal or contraluminal surfaces of intestinal tissues. Epithelial cells in this system retain viability and active transport properties for periods >3 h (Söderholm et al. 1998). Alternative systems for measuring bacterial interactions with mucosal tissue, such as in vitro organ culture techniques (Hicks et al. 1996), rely on static interactions between bacteria and cells, and have often neglected to confirm the viability of mucosal epithelial cells after several hours or days in culture.

3.3.1 Norepinephrine and Dopamine, Their Receptors, and Bacterial Pathogen Invasion in the Small Intestinal Mucosa

In Peyer's patches from the porcine small intestine, the invasion of enteropathogenic bacteria to the mucosa of the small intestine appears to be modulated by the enteric nervous system. Inhibition of neural conduction by the serosal side addition of the neuronal sodium channel blocker saxitoxin increases internalization of luminally-inoculated *Salmonella enterica* serovar Choleraesuis and EHEC by greater than sixfold in Peyer's patch explants from the porcine jejunum. Internalization of a rodent commensal *E. coli* strain is unaffected by the toxin (Green et al. 2003) and that of *S. enterica* serovar Typhimurium is decreased by threefold (Brown and Price 2008).

The serosal application of 10 μM NE produced a six- to ninefold increase in luminal *S. enterica* serovar Choleraesuis and EHEC internalization in porcine Peyer's patch explants. This effect was abolished in tissues pretreated with the

alpha-AR antagonist phentolamine. Luminally-applied NE had no effect. Thus, this action of NE appears to be mediated by *alpha*-ARs that are likely localized to the basolateral aspect of Peyer's patch epithelial cells (Green et al. 2003). In a study of *S. enterica* serovar Typhimurium internalization, the serosal application of DA or the sympathomimetic drugs cocaine and methamphetamine (which block the NE transporter, see above) decreased Salmonella recovery from Peyer's patch explants (Brown and Price 2008). Although these neurally-mediated effects on *Salmonella* internalization may appear to be small, it should be emphasized that they were measured over a surface area of 2 cm^2 in isolated tissues in a relatively short (90 min) time period. If extrapolated across the large surface area encompassed by the small intestine or even to the total area representing the Peyer's patch epithelium, these changes in Salmonella uptake may be of considerable translational significance. At present, it is not known if these catecholamine effects occur in species other than swine, and studies of this phenomenon should be extended to other animal species and bacterial strains, including those represented in the gut microflora.

Electrical stimulation of enteric nerves in explants of non-Peyer's patch mucosa from porcine jejunum more than doubled *S. Typhimurium* internalization, and neural conduction blockade decreased *S. Typhimurium* internalization by three- to four-fold. Pretreatment of explants with phentolamine also inhibited the electrically-induced increase in *S. Typhimurium* internalization (Schreiber et al. 2007).

3.3.2 Norepinephrine and Bacterial Adherence to the Cecal and Large Intestinal Mucosa

Norepinephrine and DA are equipotent in their ability to increase the number of EHEC adhering to the mucosal surface of cecal explants from mice. Their effects are prevented by AR and DAR antagonists respectively, suggesting that they are mediated by specific catecholamine receptors (Chen et al. 2003). The concentrations of NE applied to the basolateral aspect of the intestinal epithelium that are sufficient to promote EHEC adherence are lower than those necessary to promote epithelial EHEC adherence when incubated directly with the bacterium (Vlisidou et al. 2004; Bansal et al. 2007). Thus, the mechanisms underlying the mucosally-directed actions of catecholamines on EHEC adherence seem to differ from those responsible for the direct effects of NE on EHEC adherence (Freestone et al. 2007).

In mucosal explants of porcine cecum and colon, NE interacts with $alpha_2$-ARs to increase mucosal adherence of EHEC (Green et al. 2004). In contrast, increases in active anion secretion across the porcine colonic mucosa are mediated by *alpha1-ARs* (Traynor et al. 1991). Therefore, it appears that the actions of NE on ion transport and EHEC adherence are not linked through a common cellular mechanism. A$lpha_2$-ARs are negatively coupled to cyclic AMP production, which in turn leads to decreased intracellular protein kinase A activity. The adherence-promoting action of NE in the porcine colonic mucosa is inhibited by the protein kinase A activator Sp-cAMPS and mimicked by the protein kinase A inhibitor Rp-cAMPs (Green et al. 2004). From

these data, it can be hypothesized that as yet unidentified downstream substrates in mucosal epithelial cells, which are subject to protein kinase A-induced phosphorylation, act to inhibit EHEC adherence. Accordingly, a decrease in kinase activity produced by NE would reduce this phosphorylation process and increase bacterial adherence. The effects of NE in the mouse and pig cecal mucosae are rapid (≤90 min) and experiments with EHEC *eae* and *EspA* deletion mutants suggest that NE and other sympathomimetic drugs enhance early, non-intimate bacterial adherence (Chen et al. 2003; Chen et al. 2006). This is further evidence supporting an epithelium-driven interaction rather than one mediated by bacterially-derived adherence factors.

Endogenous NE secreted from nerve terminals that are present in mucosal explants appears to modulate EHEC adherence as well. The monoamine oxidase inhibitor pargyline, which inhibits NE breakdown and inhibitors of NE reuptake into nerve terminals, including desipramine and cocaine, can mimic the actions of exogenous NE and the effects of these drugs are likewise inhibited by the *alpha*-AR antagonist phentolamine (Green et al. 2004; Chen et al. 2006). Catecholamines and in particular NE may play a physiological role in promoting bacterial colonization of the large intestine, perhaps as an element of competitive exclusion. Norepinephrine increased cecal adherence of a non-O157 commensal strain of *E. coli*, which was isolated from the porcine colonic mucosa (Chen et al. 2006). One interpretation of this result is that the action of NE, which is mediated by *alpha*-ARs in the intestinal mucosa, is not limited to a particular bacterial strain or species. Clearly, this is an area requiring further investigation.

3.4 Potential Host Mechanisms That May Underlie Catecholamine-Associated Mucosal Interactions with Bacteria

3.4.1 Catecholamines and Mucosal Ion and Water Transport

Catecholamines, and in particular NE, are poised to modulate mucosal associations with bacteria, however the mechanisms underlying catecholamine actions remain to be further defined. As indicated above, the actions of catecholamines on epithelial active ion transport are well known. Norepinephrine alters active, transepithelial ion transport in the intestinal mucosa through interactions with functionally-defined *alpha*-ARs and to a lesser extent, *beta*-ARs. Its action is mediated indirectly through enteric nerves or by direct effects on epithelial cells depending upon the animal species and intestinal segment examined (Brown and O'Grady 1997; Horger et al. 1998). Dopamine alters active ion transport through direct and indirect actions on enteric ARs and DARs (Donowitz et al. 1982, 1983; Vieira-Coelho and Soares-da-Silva 1998; Al-Jahmany et al. 2004; Zhang et al. 2007, 2008, 2010; Feng et al. 2013). The delivery of mucins and SIgA, which are secreted in the intestinal crypts, to the mucosal surface

would be affected by changes in water movements across the mucosa that accompany the active transepithelial fluxes of electrolytes (Hecht 1999). As discussed below, both types of molecules contribute to the mucosal "glycobiome" (Ouwerkerk et al. 2013), which protects the mucosal surface and regulates the composition and spatial characteristics of the overlying microbial community. Moreover, in the small intestine, the movement of these proteins in the crypt to villous direction promotes sterility in the crypts, an area possessing limited barrier function and harboring a critical stem cell population involved in epithelial renewal (Johansson et al. 2013).

3.4.2 Catecholamines and Mucus Secretion

The layer of mucus coating the intestinal mucosal surface protects vulnerable epithelial cells from microbial infection, has important roles in the support of commensal microbial communities in the large intestine and, in association with active anion secretion and intestinal motility, facilitates the transit of microbes from the small intestine to the colon (Johansson et al. 2013). The principal structural components of mucus are gel-forming mucin glycoproteins, which are secreted by intestinal goblet cells (Johansson and Hansson 2013). Glycosylated molecules important for mucosal protection such as SIgA (see below) bind to carbohydrate domains in mucin and are retained in mucus layers (Meyer-Hoffert et al. 2008; Antoni et al. 2013).

In addition to secreted mucins, transmembrane mucins are expressed by enterocytes, where they contribute to the formation of an apical glycocalyx, which acts as an epithelial binding site for bacteria (Patsos and Corfield 2009). Furthermore, this class of membrane-tethered mucins may participate in cell to cell interactions and can be coupled to intracellular signaling pathways (Jonckheere et al. 2013).

Epinephrine and NE have long been known to regulate the secretion of gel-forming mucins in the airways of several animal species (but not humans) (Rogers 2002) and act through *beta*-ARs, with a smaller contribution from *alpha*-ARs, to stimulate mucin secretion from salivary glands (Quissell and Barzen 1980; Dohi et al. 1991; Taylor and McWhorter 1991). It is generally appreciated that enteric neurotransmitter substances can influence the secretion of gel-forming mucins from intestinal goblet cells (Neutra et al. 1984). However, surprisingly little is known about the regulation of this process by catecholamines. *Beta*-AR agonists have been shown to increase mucin secretion in the murine colonic mucosa (Smith and Butler 1974), but they have no effect on mucin output in rat small intestine (Mantle et al. 1991). Moreover short-term stress, which is generally associated with increased sympathetic outflow to the digestive tract, decreases mucin production in the rat colon. However, this effect remains unaltered in adrenalectomized rats or animals pre-treated with bretylium, which blocks NE release from sympathetic nerve terminals (Pfeiffer et al. 2001).

Some species of resident bacteria in the intestine express adhesins that recognize and bind to glycan residues on mucins (Etzold and Juge 2014), and some enteropathogens have developed virulence mechanisms that allow them to circumvent the

mucus barrier (Johansson et al. 2013). Catecholamines could promote epithelial invasion by Salmonella or other entero-invasive bacteria or enhance adherence of entero-adherent pathogens like EHEC by directly altering the secretion of gel-forming mucins and the thickness of the mucus covering epithelial cells. Because ARs and DARs are coupled to intracellular changes in cyclic AMP, free calcium, arrestin and other downstream signaling pathways, it is conceivable that catecholamines could alter aspects of mucin gene expression, biosynthesis or turnover (Theodoropoulos and Carraway 2007). They could also affect mucus dynamics by altering transepithelial ion and fluid transport, which would affect the hydration state and protective capabilities of intestinal mucus. Indeed, catecholamines activate cystic fibrosis transmembrane regulator (CFTR) anion channels and CFTR-dependent bicarbonate transport is essential for proper mucous discharge (Joo et al. 2001; Ambort et al. 2012; Yang et al. 2013). A role of transmembrane mucins in the interactions between intestinal epithelial cells and bacteria has not been defined. Catecholamines may rapidly up-regulate particular classes of membrane-tethered mucins, which could serve as bacterial docking sites on epithelial cells. These possibilities await investigation.

In the Ussing chamber paradigm, the tangential flow of bathing media could physically disrupt mucus overlying the epithelium, thereby exposing epithelial cells to increased bacterial contact. This may occur particularly with small intestine explants that, in contrast to the large intestine, do not possess a firmly attached inner mucus layer at the mucosal surface that is relatively resistant to mechanical disruption (Johansson et al. 2013). In IVOC systems, on the other hand, mucosal specimens are reportedly covered with a thick coating of mucus, which can restrict early bacterial contact with epithelial cells (Haque et al. 2004). Because they lack goblet cells, cell culture systems including three-dimensional organotypic models do not possess visco-elastic mucus that would serve as a complicating variable in the interpretation of experiments involving epithelial-bacterial interactions (Barrila et al. 2010). Of course, due to their reduced cellular complexity, these preparations have their own limitations when one considers the several potential sites of catecholamine action in the intestinal mucosa.

3.4.3 Catecholamines and Secretory Immunoglobulin A (SIgA)

Immunoglobulin A in a dimeric form is synthesized in and secreted from plasma B lymphocytes residing in the lamina propria underlying the intestinal mucosa. The joining (J) peptide chain between the two IgA molecules serves as a ligand for the polymeric immunoglobulin receptor (pIgR), which is situated on the basolateral membrane of crypt epithelial cells and serves to transport dimeric IgA through epithelial cells and into the intestinal crypts. Dimeric IgA that has undergone

transcytosis is proteolytically cleaved from pIgR, but retains a portion of the receptor that is known as secretory component. SIgA therefore consists of dimeric IgA complexed with secretory component (Brandtzaeg et al. 2008). Once delivered by ion and water fluxes to the mucosal surface, these molecules can bind to and distribute within the intestinal mucus and bind as well to bacteria because of their highly-glycosylated chemical structures. SIgA and free secretory component play important roles in mucosal protection by reducing contact of microbial pathogens with mucosal epithelial cells; decreasing mucosal inflammation, which could compromise epithelial barrier function; and regulating the gut microbiota (Corthésy 2013). They have also been found to bind to the apical glycocalyx on M cells in Peyer's patch follicles, which serve as sites for contact between luminal bacteria and the follicle-associated epithelium (Kato 1990).

Within the digestive tract, it is well established that catecholaminergic nerves act on salivary glands to regulate SIgA secretion in saliva (Proctor and Carpenter 2007). In the intestine, my collaborators and I have reported that adrenergic nerve fibers immunoreactive for dopamine *beta*-hydroxylase are present throughout the submucosa and terminate near the basal membranes of crypt and villous/surface epithelial cells of the porcine ileum, distal colon and cecum (Schmidt et al. 2007; Green et al. 2004; Chen et al. 2006). In the porcine small and large bowel mucosa, dopamine beta-hydroxylase-immunoreactive nerves terminate near IgA-positive B lymphocytes and neighboring epithelial cells that display immunoreactivity for pIgR (large intestine: Schmidt et al. 2007; small intestine, Fig. 3.1: L.D. Schmidt, L. Vulchanova and D.R. Brown, unpublished observations). Norepinephrine stimulates the vectorial secretion of secretory IgA in mucosa explants from the porcine ileum (Fig. 3.2) and colon (Schmidt et al. 2007). This effect has been attributed to an *alpha-AR-mediated* increase in the epithelial turnover of pIgR resulting in the vectorial transport of SIgA as well as free secretory component towards the mucosal surface (Schmidt et al. 2007; L.D. Schmidt, Y. Xie, L. Vulchanova and D.R. Brown, unpublished observations).

In the rat small intestine, NE enhances the expression of pIgR mRNA (Reyna-Garfias et al. 2010). Restraint stress in rats increases IgA content in the lamina propria of the small intestine, an effect inhibited by chemical sympathectomy with 6-hydroxydopamine (Reyna-Garfias et al. 2010). Sympathectomy alone has been reported to reduce gut IgA responses to oral antigen exposure in adult rats (González-Ariki and Husband 1998) and decrease the number of IgA-positive lamina propria cells in the weanling rat (González-Ariki and Husband 2000).

In humans, NE has been found to increase the number of IgA-immunoreactive cells in the intestinal wall of marathon runners (Nilssen et al. 1998). Moreover, EPI has been found to increase pIgR expression and promote mucosally-directed SIgA transport in polarized monolayers of human airway epithelial cells (Calu3). However, NE or a selective $beta_2$-AR agonist had no effect on SIgA transport in this cell line (Ali et al. 2014).

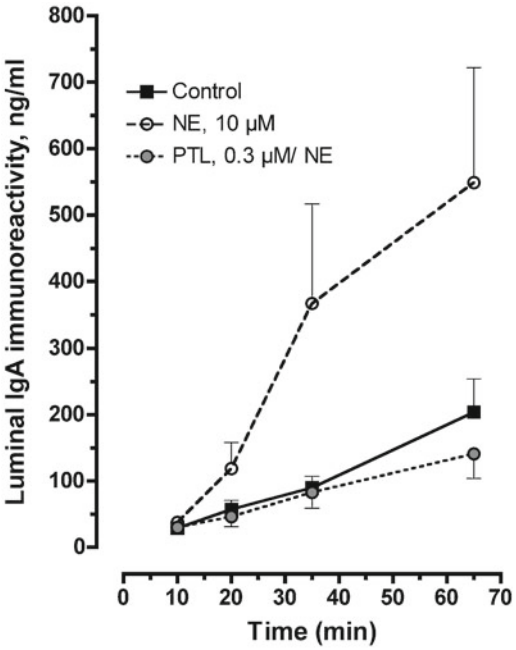

Fig. 3.2 Effect of norepinephrine (NE) on luminal IgA secretion from explants of porcine ileal mucosa. Tissues were treated with NE (10 μM) in the absence or presence of the *alpha*-adrenergic antagonist phentolamine (PTL) at a concentration of 0.3 μM in the contraluminal bathing medium. Tissues untreated with drugs served as controls in each experiment. NE was added to the contraluminal bathing medium 5 min after the start of each experiment (i.e. time = 5 min), and phentolamine was added immediately after collection of a luminal fluid sample at time = 0 min. Mean IgA immunoreactivity at 0 min was 92 ± 27, 80 ± 29 and 42 ± 10 ng/mL from control, NE-treated and PTL-/NE-treated tissues (n = one tissue from each of 5–7 pigs), respectively. The line elevation for NE alone was significantly different from control ($F = 17.7$, $P = 0.02$) as determined by linear regression analysis

3.4.4 Catecholamines and Other Potential Binding Sites for Bacteria on Epithelial Cells

There are potentially numerous and as yet unknown membrane proteins that could be rapidly up-regulated after catecholamine receptor activation and serve as microbial docking sites. We will discuss two classes of proteins that might be considered attractive examples of potential targets for future investigation. The first of these, $beta_1$-integrins, are heterodimeric complexes of glycoprotein subunits that are expressed on epithelial cell surfaces and have been recently found to act as MAMP receptors for some bacteria (Thinwa et al. 2014). One member of this family, CD29, has been implicated in some aspects of EHEC adherence (Sinclair et al. 2006). Heparin, which blocks epithelial $beta_1$-integrins, inhibits EHEC adherence to human colonic

epithelial cells (Gu et al. 2008). Norepinephrine stimulates the rapid up-regulation of *beta*$_1$-integrins to enhance adhesive interactions between blood cells or tumor cells with the vascular endothelium (Levite et al. 2001; Butta et al. 2004; Delahunty et al. 2006; Strell et al. 2012). Whether NE acts in a parallel fashion at mucosal sites to promote epithelial cell interactions with luminal bacteria is unknown.

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are immunoglobulin-like glycoproteins that are expressed in epithelial cells, including those of the intestine. They participate in epithelial growth and cytoplasmic signaling, but have been exploited by some pathogenic bacteria to serve as apical membrane docking and invasion sites, depending on the host species (Tchoupa et al. 2014). The effects of catecholamines or their associated intracellular signaling pathways on the expression of this class of epithelial glycoproteins and other potential target proteins on epithelial cell membranes remain to be investigated.

3.5 Conclusions

Catecholamines that are synthesized in and released from host cells residing in the intestinal tract and potentially other mucosal immune sites in the body appear to modulate interactions between epithelial cells and bacteria at the mucosal surface. Initial studies of NE effects on intestinal mucosa explants to increase bacterial adherence or invasion indicate that these phenomena extend across two mammalian species (mice and pigs) and are mediated by host cell to cell interactions within the mucosa and not to interkingdom signaling. It appears that the intestinal mucosal surface cannot be viewed as a mere passive field for microbial colonization, but rather may be able to select microorganisms with which it will associate much like the characteristic feature of mucosal sites to tolerate potentially harmful antigens and pathobionts. These investigations have been limited to mainly Gram-negative, facultative anaerobic bacilli that act as enteropathogens (*E. coli* and *S. enterica*). It remains to be determined if similar associations between the gut mucosa and other types of bacteria occur in tissues pretreated with NE or other catecholamines. Perhaps the enteropathogens that have been studied have simply co-opted epithelial processes for sampling or co-existing with beneficial microbes? The biological significance of NE-mediated mucosa:bacteria interactions clearly remains tentative at this point in time.

Based on the information available, many more questions can be asked. For example, does endogenous NE (and potentially DA) act to regulate aspects of bacterial sampling at mucosal immune recognition and processing sites, as in intestinal Peyer's patches? Are catecholamines in the intestinal mucosa and other mucosal sites involved with the process of establishing and maintaining mucosa-associated microfloral populations? What are the molecular and cellular mechanisms underlying the effects of catecholamines on epithelial cells and other cell types in the mucosa? Does psychological or physical stress or other conditions associated with increased sympathetic outflow alter the host's microbiota or increase host susceptibility to mucosal

infections? Sympathetic hyperactivity has been implicated in intestinal inflammatory conditions, and the relationship between sympathetic activity with mucosal immunity and inflammation, bacterial colonization, or the risk of mucosal infection may offer fruitful areas for investigation (Lyte et al. 2011; Verbrugghe et al. 2012; Bellinger and Lorton 2014; Bailey 2014; Mayer et al. 2015).

In addition to dissecting the cellular and molecular mechanisms underlying this phenomenon, studies of catecholamine action on bacterial adherence *in vitro* should be extended to investigations of the role of endogenous and exogenous DA and NE in isolated intestinal loops and intact animal models which encompass larger surface areas and have greater translational relevance. Studies conducted at different levels of cellular complexity may accordingly yield complex results. For example, the effects of NE on SIgA secretion seem to differ in isolated mucosa explants and ex vivo organ preparations from the same host species (Schmidt et al. 1999; Schmidt et al. 2007).

Finally, do interkingdom signals play a role in catecholamine-mediated mucosal function? Based on the current literature, there are faint, but intriguing hints that this may be so. Although it is not yet known if bacteria are capable of producing catecholamines, a bacterium has been discovered that expresses tyrosine hydroxylase-like activity, the rate-limiting step in catecholamine synthesis (Connor et al. 2011). Moreover, some short-chain fatty acids, e.g. butyrate that are produced after fermentation by commensal bacteria in the colon have been found to regulate tyrosine hydroxylase activity in a mammalian cell line (Nankova et al. 2014). Further developments along these research lines are eagerly awaited.

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Chapter 4
Dietary Catechols and their Relationship to Microbial Endocrinology

Neil Shearer and Nicholas J. Walton

Abstract This chapter examines the evidence that the ability of neuroendocrine hormones, notably norepinephrine and epinephrine, to stimulate bacterial growth in iron-restricted media is not limited to molecules with a catecholamine structure but is also possessed by a variety of other catechols, many of which are of plant origin and are common in the diet. Catechols derived from the diet, such as the tea flavanols, can be present in the plasma at submicromolar and micromolar concentrations, comparable with the concentrations of catecholamines that have been shown to be effective in promoting bacterial growth under conditions of iron restriction, although many dietary catechols, notably quercetin derivatives, are present in the plasma and tissues largely as conjugates, from which the catechol function has been lost. Finally, although bacterial growth promotion through relief of iron restriction appears to be exhibited by a wide range of catechols, the gene-activation effects of catecholamines demonstrated to occur in some bacteria may be much more specific, although the definitive experiments to establish structure-function relationships have yet to be reported.

4.1 Introduction

The effects of catecholamines on bacterial growth and behaviour are well documented (see other chapters in this volume) and appear to occur principally through facilitation of iron acquisition from the host glycoproteins, transferrin and lactoferrin. Nevertheless, it is clear that mechanisms other than simple iron acquisition are responsible for some of the effects of catecholamines. The question that naturally arises, however, is how far these phenomena are more generally characteristic of compounds with a catecholic structure, rather than being specific to the neuroendocrine catecholamines, norepinephrine (noradrenaline), epinephrine (adrenaline) and

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dopamine, or their analogues. This is important, since catechols are common; in particular, they are ingested routinely as components of plant-based diets and often in substantial amounts. A number of subsidiary questions then arise: what are these other catecholic compounds and what are the prevalent dietary sources; what levels of these compounds or of their relevant metabolites might exist in plasma, organs or tissues; and are there any specific effects of catecholamines upon bacteria that are not observed with other catechols, and vice versa? This account attempts to provide some insights.

4.2 Dietary Sources and Distribution of Catechols

Catechols bind iron, and this property is exploited in catecholic siderophores (Crosa and Walsh 2002) and, by contrast, in strategies to prevent bacterial growth by restricting the supply of iron (Scalbert 1991; Mila et al. 1996). Further, since free Fe^{2+} ions participate in the Fenton reaction with H_2O_2, which produces highly reactive hydroxyl radicals, the sequestration of free iron by catechols and other iron-chelating compounds can restrict the potential for free-radical generation and protect biological molecules from oxidative damage (Rice-Evans et al. 1995; Khokhar and Apenten 2003). Catechols also possess an autoxidation activity that oxidises Fe^{2+} to Fe^{3+}, although conversely, reduction of Fe^{3+} to Fe^{2+} can also occur, particularly at low pH (Moran et al. 1997; Chvátalová et al. 2008). Catechols with an additional neighbouring hydroxyl group, such as gallic acid (3,4,5-trihydroxybenzoic acid), appear to bind iron and also to oxidise Fe^{2+} to Fe^{3+} somewhat less effectively than simple catechols such as protocatechuic acid (3,4-dihydroxybenzoic acid) (Khokhar and Apenten 2003; Andjelković et al. 2006; Chvátalová et al. 2008). As discussed later, a study of the effects of tea catechins revealed that only compounds possessing a 3,4-dihydroxyphenyl group in the B-ring stimulated bacterial growth under iron-restrictive conditions; compounds with an additional neighbouring hydroxyl group in the 5-position failed to stimulate growth (Freestone et al. 2007c). In this chapter, the term catechol is used to denote compounds with the *o*-dihydroxyphenyl, as distinct from a trihydroxyphenyl, grouping.

Catechols in the diet are most often plant-derived. The large majority are eventual products of the central phenylpropanoid pathway from phenylalanine via cinnamic acid. In plants, phenylpropanoid-pathway derivatives fulfil diverse functions in defence, signalling, protection against ultraviolet light and insect attraction (Parr and Bolwell 2000; Boudet 2007). Those present in the largest amounts are likely to serve a broad-spectrum defence function as anti-feedants or as antimicrobial agents. Catechols are rarely a specific focus of attention, being more often considered non-specifically within other secondary-product classes, especially the hydroxycinnamic acids and the various sub-classes of the flavonoids. Many plant polyphenolic substances exhibit a broad-spectrum antibacterial activity and catechols are not necessarily among the most potent (Taguri et al. 2006); however, studies of sidero-

phore mutants of *Erwinia chrysanthemi* demonstrate that plant polyphenols containing catechol groups can act to prevent bacterial growth by sequestering Fe^{3+}, although sequestration of other metal ions, notably Cu^{2+} and Zn^{2+}, might also occur (Mila et al. 1996). As discussed further, plant catechols include, in particular, catecholic representatives of the benzoic and cinnamic acids and their derivatives, notably protocatechuic acid (3,4-dihydroxybenzoic acid), caffeic acid (3,4-dihydroxy-*trans*-cinnamic acid) and the chlorogenic acids (principally 5-*O*-caffeoylquinic acid); oleuropein, a hydroxytyrosol ester found in olives; catecholic flavonols, notably quercetin; catecholic flavanols, for example (−)-epicatechin and (−)-epicatechin gallate; many anthocyanins, which are glycosides of anthocyanidins such as the catechol, cyanidin, and which are widespread as blue, purple and red plant pigments; and finally, many proanthocyanidins or condensed tannins, formally polymerised flavanols. Flavonols, flavones and anthocyanins, though not flavanols, are all generally not found free in plants but are typically found as *O*-glycosides. Figure 4.1 shows the structures of the principal flavonoid sub-classes.

Many of these compounds attract considerable interest on account of their reported effects in relation to a range of cancers, inflammatory conditions and cardiovascular diseases, which to varying extents (and not in every case) may be a result of their radical-scavenging and antioxidant properties (Ross and Kassum 2002; Cooper et al. 2005; Evans et al. 2006; Hodgson and Croft 2006; Prior et al. 2006; Rahman 2006; Schroeter et al. 2006; Espín et al. 2007; Khan et al. 2008; Loke et al. 2008). One consequence of this is a concern to acquire reliable data on dietary intakes. Extensive information on the contents of phenylpropanoid metabolites in fruits, vegetables and beverages has been compiled (Hollman and Arts 2000; Tomás-Barberán and Clifford 2000; Clifford 2000a, b; Santos-Buelga and Scalbert 2000; Manach et al. 2004). Levels can vary widely on account of varietal characteristics, cultural conditions, developmental stage, position on the plant and storage – and very often within an individual harvested fruit or vegetable (Manach et al. 2004).

Catecholic benzoic and cinnamic acids are amongst the simplest catecholic compounds found in plant foods. They and their derivatives are widespread and may sometimes be present in appreciable amounts. Thus, blackberry fruits may contain ca. 0.07–0.2 g of protocatechuic acid kg^{-1} fresh weight (Tomás-Barberán and Clifford 2000) and potato tubers may contain around 1.2 $g\,kg^{-1}$ fresh weight of chlorogenic acid (principally 5-*O*-caffeoylquinic acid), though much is likely to be lost during cooking (Clifford 2000a). Coffee also contains large amounts of chlorogenic acid and Clifford (2000a) has estimated that 200 ml of instant coffee brew (2% w/v) may provide 50–150 mg of the compound (equivalent to ca. 25–75 mg of caffeic acid).

The occurrence of oleuropein, a secoiridoid glucoside ester of the catechol, hydroxytyrosol, is restricted to olives, where in young fruits it may account for 14% of dry matter, although in processed fruit and in olive oil, levels are lower than this, partly on account of hydrolysis, including hydrolysis to hydroxytyrosol. Olives also contain verbascoside, a compound containing two catecholic residues, hydroxytyrosol and caffeic acid (Soler-Rivas et al. 2000).

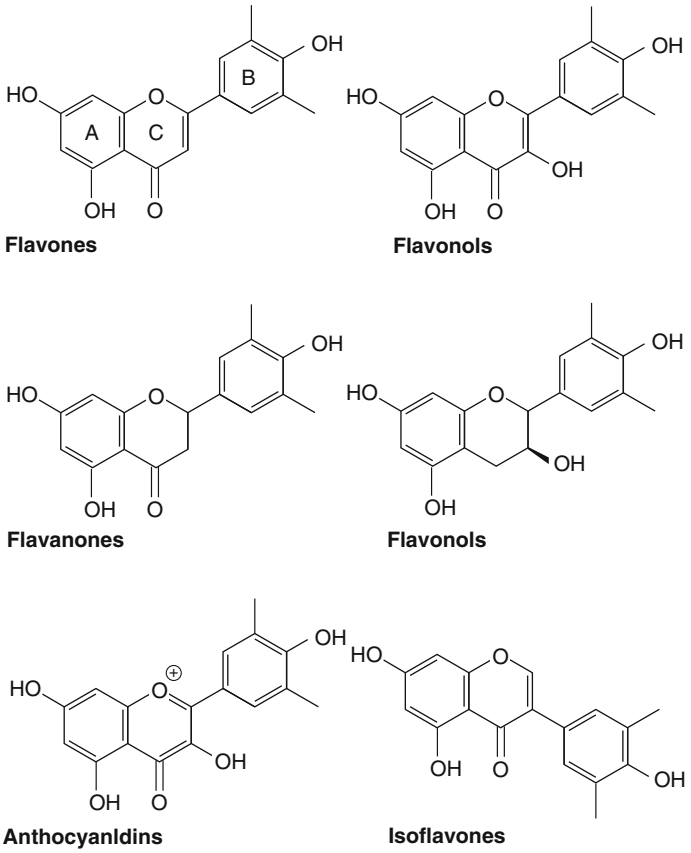

Fig. 4.1 Subclasses of the flavonoids. Classification is based on variations in the heterocyclic C-ring. Reproduced from Hollman and Arts (2000), © Society of Chemical Industry, with permission

In most fruits, vegetables and beverages the levels of flavonols, flavones and flavanols are below about 0.015 g kg^{-1} fresh weight, although there are conspicuous exceptions (Hollman and Arts 2000). Thus, the catecholic flavonol, quercetin, occurs in the form of glycosides in onions at 0.35 g kg^{-1} fresh weight and in kale at around 0.11 g kg^{-1} fresh weight (and in each case possibly considerably more, depending upon variety and cultural conditions); the flavone, luteolin, may reach 0.2 g kg^{-1} fresh weight in celery leaves; levels of the catecholic flavanols, (−)-epicatechin and (−)-epicatechin gallate, can reach ca. 20–150 μg ml^{-1} in brewed tea; and levels of (+)-catechin and (−)-epicatechin can reach 100–200 μg ml^{-1} in some red wines. Cocoa and chocolate are also rich sources of flavanols (Schroeter et al. 2006). Levels of anthocyanins are greatest in those fruits and vegetables that are highly pigmented; for example, blueberries contain ca. 0.8–4.2 g of anthocyanins kg^{-1} fresh weight and aubergines may contain 7.5 g kg^{-1} fresh weight (Clifford 2000b). Not surprisingly, red wines, and port wine in particular, contain appreciable

levels of anthocyanins, within the range 140–1,100 μg ml^{-1} (Clifford 2000b). Proanthocyanidins or condensed tannins are abundant in many common or staple foods or beverages (Santos-Buelga and Scalbert 2000), notably black tea, in which two groups of these compounds, the theaflavins and thearubigins, are derived from the flavanols of green tea during processing. Levels of proanthocyanidins of ca. 3–10 g kg^{-1} dry weight in lentils, of up to 7.4 g kg^{-1} dry weight in faba beans and of up to 39 g kg^{-1} dry weight in sorghum, have been determined. High concentrations of proanthocyanidins are often present in red wine and apple juices, and particularly in cider, where levels are reported to range between 2,300 and 3,700 μg ml^{-1} (Santos-Buelga and Scalbert 2000).

In addition to the catechols discussed above, catecholamines also occur in many plants, and there is evidence for their involvement in defence against pathogens, in responses to plant growth substances and in carbohydrate metabolism, but details of the mechanisms involved still remain uncertain (Kulma and Szopa 2007). Where determined, levels of catecholamines have been found to be low (below 1 mg kg^{-1} fresh weight), except for bananas, plantains and avocados. Thus, levels in excess of 40 mg kg^{-1} of dopamine were found in the fruit pulp of red banana and yellow banana, whilst the peel of Cavendish banana contained 100 mg kg^{-1} (Kulma and Szopa 2007). These values are similar to those of, for example, protocatechuic acid in blackberry fruits (Tomás-Barberán and Clifford 2000). Fruit pulp of Fuerte avocado contained smaller amounts of catecholamines: 4 mg kg^{-1} of dopamine and <3.5 mg kg^{-1} of norepinephrine. The relatively high content of dopamine in banana pulp is of particular interest in view of the ability of banana pulp to promote the growth of Gram-negative bacteria in iron-restricted medium, as reported by Lyte (1997).

Dietary catechols also arise from non-plant sources. In particular, tyramine arises in cheeses and other fermented foods as a result of the bacterial decarboxylation of tyrosine (Santos 1996). Concentrations exceeding 0.1 g kg^{-1} can be present in matured cheeses (Komprda et al. 2008). Micromolar concentrations of tyramine have been found to increase the adherence of *Escherichia coli* O157:H7 to murine intestinal mucosa (Lyte 2004b).

4.3 Dietary Intakes of Catechols

Manach et al. (2004) reviewed and summarised data from several countries, including the United States, Denmark, the Netherlands and Spain and concluded that the total intake of polyphenols probably reached 1,000 mg d^{-1} in individuals who ate several portions of fruit and vegetables each day. The consumption of flavonols probably accounted for 20–50 mg d^{-1}, only a proportion of which would be the glycosides of quercetin and other catechols. Levels of anthocyanins consumed were estimated to be broadly similar (though somewhat higher in Finland where appreciable amounts of berries are commonly eaten). However, depending

upon individual dietary habits, the intakes of both total and specific polyphenols are likely to be highly variable. In some individuals, chlorogenic acid may predominate as a result of coffee consumption and could amount to more than 200 mg d^{-1} (Clifford 2000a), whereas drinkers of tea may well ingest comparable amounts of catecholic flavanols and proanthocyanidins (Hollman and Arts 2000; Santos-Buelga and Scalbert 2000; Cooper et al. 2005). Taking all principal sources into account, daily intakes of catechols therefore seem likely to exceed 500 mg d^{-1} in some individuals.

4.4 Absorption and Availability of Catechols

Although substantial amounts of catechols may be consumed in the diet, many intervening factors and processes will determine whether appreciable concentrations are to be found in the plasma and in tissues. These include the structure of the particular compound, notably the extent and type of glycosylation or esterification; the extent and mechanisms of absorption; the degree of conjugation or derivatisation during or following absorption; potential food-matrix effects; the extent of any metabolism by the gastrointestinal flora, dependent in part upon the degree to which the compound remains unabsorbed (or is possibly re-secreted) in the small intestine; and finally, absorption and metabolism by individual tissues and organs (Manach et al. 2004, 2005; Prior et al. 2006).

Structural effects on absorption are readily apparent, for example, in the case of quercetin glycosides. The 4′-glucoside of quercetin is absorbed more rapidly than the 3β-rutinoside (rutin); maximal absorption of the former occurs about 30–40 min post-ingestion in humans, whereas maximal absorption of the latter requires 6–9 h. Thus, quercetin is found to be absorbed more rapidly from onions, which contain the flavonol predominantly in the form of glucosides, than from apples, which contain it in the form of both glucosides and other glycosides (Hollman et al. 1997). Enzyme systems of the small intestine that can perform the uptake and hydrolysis of quercetin glucosides have been identified and comprise the sodium-dependent glucose transporter, SGLT1, followed by a cytosolic β-glucosidase, or alternatively lactase phorizin hydrolase, a glucosidase of the brush border membrane (Day et al. 2003; Németh et al. 2003; Sesink et al. 2003); glycosides other than glucosides may be absorbed relatively poorly in the small intestine and absorption may be appreciably dependent upon bacterial hydrolysis in the colon. In contrast to flavonols, flavanols are not glycosylated and therefore require no hydrolysis step (Manach et al. 2004). Simple hydroxybenzoic or hydroxycinnamic acids, such as caffeic acid, also appear to be readily absorbed (Clifford 2000a; Cremin et al. 2001), although chlorogenic acid (5-*O*-caffeoylquinic acid) is not, and hydrolysis by a number of colonic bacteria (strains of *E. coli*, *Bifidobacterium lactis* and *Lactobacillus gasseri*) and by human faecal microbiota has been demonstrated (Couteau et al. 2001; Gonthier et al. 2006). Anthocyanins differ from flavonols in that the native glycosides can

apparently be absorbed and appear in the plasma, although bioavailability appears comparatively low (Wu et al. 2002; Manach et al. 2005). The major metabolite of cyanidin glucosides in humans is protocatechuic acid (Vitaglione et al. 2007). Proanthocyanidins, particularly those of higher molecular weight, are poorly absorbed, and large proportions are likely to reach the colon (Manach et al. 2004, 2005).

Following absorption, most catechols and polyphenols become derivatised by glucuronidation, sulphation or methylation, or by a combination of these, which are reactions that are undergone by many xenobiotics (Wu et al. 2002; Manach et al. 2004, 2005; Prior et al. 2006). The UDP-glucuronosyltransferase, sulphotransferase and catechol-*O*-methyltransferase (COMT) activities that are responsible are active in intestinal enterocytes and in the liver; COMT is also active in kidney and has a wide tissue distribution. Prior et al. (2006) have summarised the principal patterns of conjugation that are observed for flavonoids. Thus, flavonoids with a catecholic B-ring, such as quercetin and cyanidin, are derivatised mainly to 3′-*O*-methyl derivatives, with smaller amounts of 4′-*O*-methyl derivatives. The catecholic B-ring also promotes glucuronidation. For quercetin, the principal compounds detected in human plasma after the ingestion of quercetin glucosides contained in onion were found to be 3′-*O*-methylquercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulphate (Day et al. 2001). Extensive methylation and glucuronidation of the 3′- and 4′-positions also occur in the case of catechin and epicatechin (Natsume et al. 2003).

The bioavailability of polyphenols, including their pharmacokinetics in plasma has been comprehensively reviewed (Manach et al. 2005). For example, meals containing the equivalent of 80–100 mg of quercetin gave rise to plasma concentrations of quercetin conjugates of around 0.3–0.75 μM (Hollman et al. 1997; Manach et al. 1998; Graefe et al. 2001); consumption of 80 g of chocolate (containing a total of 5.3 mg of procyanidins g^{-1}) could produce a plasma epicatechin concentration of as much as 0.36 μM (Rein et al. 2000; Wang et al. 2000); and a glass of red wine containing 35 mg of catechin could produce a plasma catechin concentration of 0.09 μM (Donovan et al. 1999). As reviewed by Manach et al. (2004, 2005), half-lives in plasma have been found to be variable, from as little as ca. 2 h for anthocyanins and flavanols to 11–28 h for quercetin derivatives. Therefore, unless dietary catechols are ingested at frequent intervals, their plasma levels, or those of their derivatives, may decline rapidly, and only in the case of compounds such as quercetin glycosides can successive intakes lead to some degree of accumulation of plasma derivatives (Manach et al. 2005). Elimination by both biliary and urinary routes has been demonstrated, with the biliary route likely to be predominant for larger and multiple-conjugated metabolites (Manach et al. 2004).

Besides appearing in plasma, labelled dietary phenolic compounds can give rise to derivatives in a wide range of tissues and organs, especially the digestive organs and the liver. From a number of studies in different laboratories, levels in rats and mice varied between 30 ng and 3,000 ng (as aglycone) g^{-1} of tissue, depending upon the compound and the dose administered (Manach et al. 2004). It is likely that accumulation in any given organ is non-homogeneous, although it is not clear to what extent organ- or tissue-specific uptake mechanisms may exist.

4.5 Bacterial Growth May be Stimulated Experimentally by a Range of Catechols

It is clear that catechols ingested in the diet, or their derivatives, may be found in plasma and tissues at submicromolar and micromolar levels. These are comparable with, or higher than, normal plasma concentrations of norepinephrine and epinephrine, which are in the nanomolar range (Benedict and Grahame-Smith 1978); and, against this background, we shall shortly examine the evidence from two studies (Coulanges et al. 1998; Freestone et al. 2007c) that have shown that dietary catechols can stimulate bacterial growth in a manner similar to that observed with the neuroendocrine catecholamines, norepinephrine and epinephrine.

However, it is important to keep in mind that a substantial proportion, possibly approaching 50% in some cases (see Prior et al. 2006), of dietary catechol derivatives in the plasma and tissues may be conjugates, in which one of the catecholic hydroxyl groups is methylated, glucuronidated or sulphated. Furthermore, these compounds may not be free in solution. Quercetin incubated with human plasma becomes almost entirely bound to plasma proteins, chiefly albumin (Boulton et al. 1998) and other flavonoids behave similarly, although sulphation and glycosidation may substantially reduce the binding affinity (Dufour and Dangles 2005). The potential effect of protein binding is important because the stimulation of bacterial growth in iron-restricted medium that occurs in response to norepinephrine appears to occur concomitantly with uptake of the catecholamine into the bacterial cell (Freestone et al. 2000). However, the role of norepinephrine uptake in relation to both iron uptake and growth stimulation still remains unclear (Freestone et al. 2003, 2007b). It is not yet known whether the iron uptake and growth stimulation that occur in response to dietary catechols, and which are discussed below, are associated with the bacterial uptake of these compounds. In any event, it remains to be established how far the reported effects of catechols on bacterial growth and behaviour are affected by protein-binding of the catechol. However, the iron-restricted, serum-SAPI medium employed by Freestone et al. (2000, 2007c) contains, by definition, the proteins present in adult bovine serum, suggesting that the binding of catechols to proteins is not an issue, at least in this experimental set-up.

In the first of the two studies examining the effects of catechols on bacterial growth, Coulanges et al. (1998) examined the growth-promoting effects of a range of catechols upon a number of *Listeria* species, which (as far as is known) are unable to biosynthesise siderophores. Their ability to overcome growth inhibition induced by the iron-chelator, tropolone, was measured in disk diffusion assays. A number of compounds possessing a catechol grouping were effective in relieving growth inhibition. These included dopamine, epinephrine and norepinephrine and DL-DOPA, the siderophores pyoverdine and rhodotorulic acid, and plant-derived catechols including caffeic acid, esculetin, quercetin and rutin. Salicylic acid (*o*-hydroxybenzoic acid) was ineffective, as was dihydroxybenzoic acid (the isomer was not specified). This study therefore demonstrated that the relief of tropolone-induced growth inhibition in *Listeria monocytogenes* that had

previously been observed to occur with catecholamines (Coulanges et al. 1997) was not restricted to these compounds but could occur also with a range of non-amine catechols.

In the second study, and following work summarised by Freestone et al. (2002) and Lyte (2004a), Freestone et al. (2007c) examined the growth of *E. coli* O157:H7 and *Salmonella enterica* SV Enteriditis in response to a number of catechols commonly consumed in the diet, and to fruit and vegetable extracts known to contain catechols. In the case of the individual catechols, they determined growth responses both in iron-restricted medium and in iron-replete medium and followed up these experiments with measurements of the uptake of ^{55}Fe from ^{55}Fe-labelled transferrin and lactoferrin. Marked differences in growth were observed, depending upon whether a rich medium (Luria broth) or an iron-restricted medium (serum-SAPI) was employed (Figs. 4.2 and 4.3). In the rich medium, none of the compounds tested – catechin, caffeic acid, chlorogenic (5-*O*-caffeoylquinic) acid and tannic acid – had any significant effect upon growth, with the exception of tannic acid, which became progressively more inhibitory to growth with increasing concentration (up to 200 μg ml^{-1}). In contrast, in the iron-restricted medium (and hence broadly consistent with the findings in *Listeria* of Coulanges et al. 1998), all four catechol compounds tested promoted growth, by a factor of around 4 log-orders, with saturation occurring at 50–100 μM (50 μg ml^{-1} for tannic acid). Similarly, all the fruit and vegetable extracts tested (apple, carrot, grape, pear, plum, orange and strawberry), and infusions of tea and coffee, promoted growth in the iron-restricted medium. Study of the effects of tea flavanols (see Fig. 4.4) revealed that the ability to promote growth in the iron-restricted medium was restricted to those compounds possessing a 3,4-dihydroxyphenyl (i.e. catecholic) B-ring ((+)–catechin, (−)-catechin gallate and (−)-epicatechin gallate); trihydroxy-compounds with an additional hydroxyl group in the 5-position of the B-ring ((−)-epigallocatechin, (−)-epigallocatechin gallate and (−)-gallocatechin gallate) failed to promote growth.

The work of Freestone et al. (2007c) revealed important mechanistic similarities between the responses to dietary catechols and the responses to neuroendocrine catecholamines. All of the catechols and plant extracts that promoted growth in the iron-restricted medium were also able to stimulate the uptake of ^{55}Fe from ^{55}Fe-transferrin or ^{55}Fe-lactoferrin in both organisms studied (Fig. 4.5). Their activity in this respect was therefore in general comparable with that of the neuroendocrine catecholamines, typified by norepinephrine. Further similarity with the behaviour of the catecholamines was shown by the absence of any growth promotion by dietary catechols, or by fruit or vegetable extracts, in *E. coli* siderophore biosynthesis (*entA*) and transport (*tonB*) mutants. Mutations in these genes were previously shown to prevent norepinephrine-stimulated growth of *E. coli* (Burton et al. 2002; Freestone et al. 2003).

The evidence from these studies by Freestone et al. (2007c) and Coulanges et al. (1998) therefore show conclusively that the behaviour of the catecholamines in promoting growth in iron-restricted medium is not unique but instead is common to a diverse range of compounds all possessing the catechol structure. Thus, any consideration of the role of catecholamines in the promotion of bacterial growth

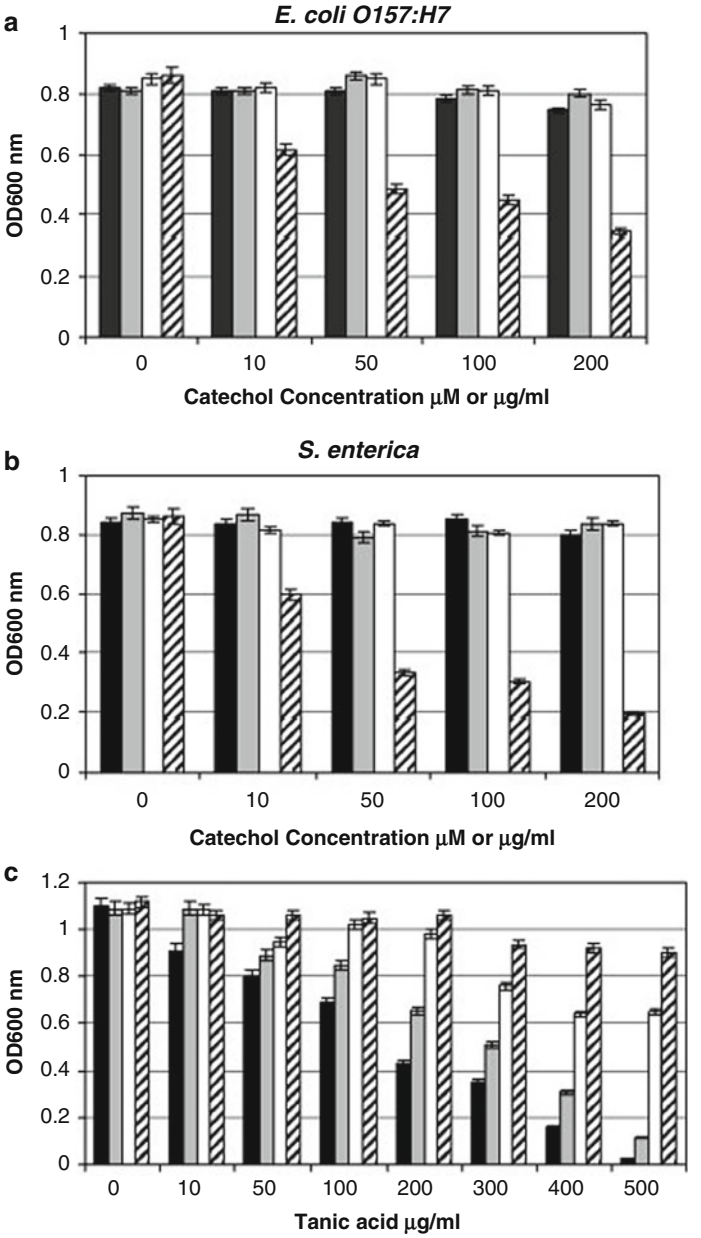

Fig. 4.2 Growth modulation by dietary catechols in laboratory culture media. *E. coli* O157:H7 (**a**) and *S. enterica* (**b**) were inoculated at approximately 10^2 CFU ml^{-1} into duplicate 1 ml volumes of Luria broth containing the concentrations of the catechols shown, incubated for 10 h and enumerated for growth by measurement of the absorbance of the cultures at 600 nm. Catechin, caffeic and chlorogenic acids are measured in μM units, while tannic acid is measured in units of μg ml^{-1}. The results shown are representative data from two separate experiments; data points showed

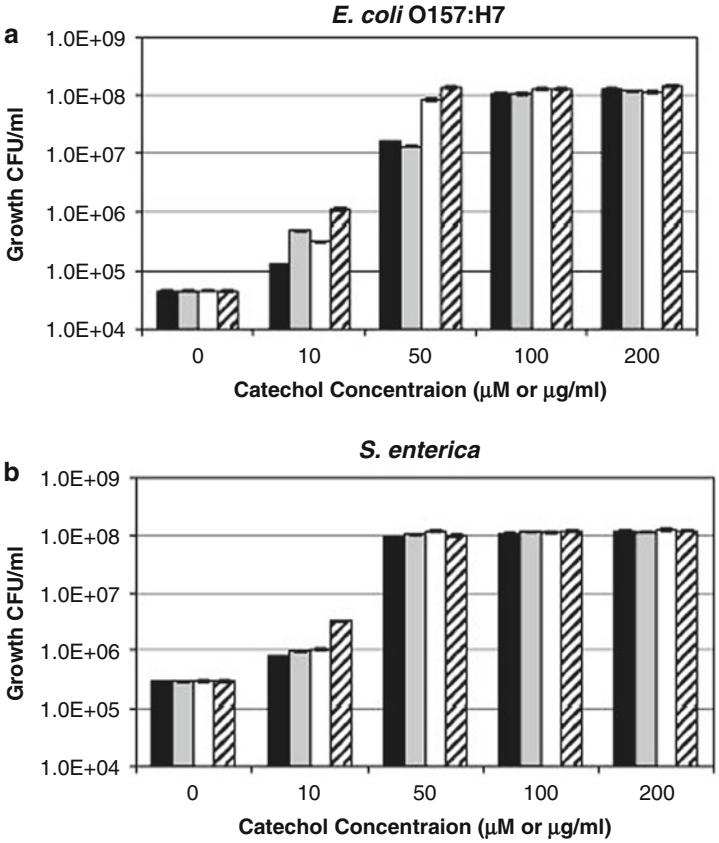

Fig. 4.3 Growth modulation by dietary catechols in serum-based media. *E. coli* O157:H7 and *S. enterica* were inoculated at approximately 10^2CFU ml^{-1} into duplicate 1 ml aliquots of serum-SAPI containing the concentrations of the catechols shown, incubated for 18 h, and enumerated for growth (CFU ml^{-1}). Catechin, caffeic and chlorogenic acids are measured in μM units, while tannic acid is measured in units of μg ml^{-1}. The results shown are representative data from two separate experiments; data points showed variation of less than 5%. Catechin (*black bar*); Caffeic acid (*grey bar*); Chlorogenic acid (*white bar*); Tannic acid (*diagonal hatch*). Reproduced from Freestone et al. (2007c), with permission

Fig. 4.2 (continued) variation of less than 5%. Catechin (*black bar*); Caffeic acid (*grey bar*); Chlorogenic acid (*white bar*); Tannic acid (*diagonal hatch*). (**c**) examines the mechanistic basis of the growth inhibition by tannic acid of *E. coli* O157:H7. A similar methodology to that used in (**a**) and (**b**) was employed, except that the culture media were supplemented with either no additions (*black bar*), 50 mM Tris–HCl, pH 7.5 (*grey bar*), 100 μM ferric nitrate (*white bar*) and 50 mM Tris–HCl, pH 7.5 and 100 μM ferric nitrate (*diagonal hatch*). Cultures were corrected for absorbance due to media components. The results shown are representative data from two separate experiments; data points showed variation of less than 5%. Reproduced from Freestone et al. (2007c), with permission

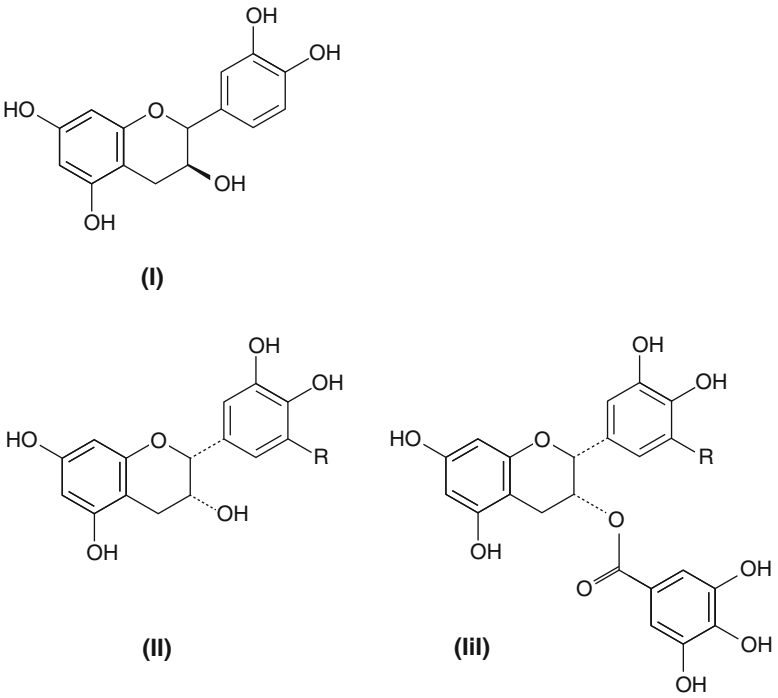

Fig. 4.4 Structures of flavanols: (+)-catechin (I), (−)-epicatechin (II, R =H), (−)-epigallocatechin (II, R = OH), (−)-epicatechin-3-gallate (III, R = H) and (−)-epigallocatechin-3-gallate (III R = OH). Reproduced from Hollman and Arts (2000), © Society of Chemical Industry, with permission

(and potentially of virulence) needs to be broadened to take account of the occurrence and effects of dietary catechols, but subject to the caveats outlined at the beginning of this section.

4.6 Are any of the Effects of Catechols on Bacteria Catecholamine-Specific?

Although a wide range of catechols are able to stimulate the growth of certain bacteria in minimal, iron-complexed media, there is at least one report of heightened specificity. The growth of *Yersinia enterocolitica* is stimulated by norepinephrine and dopamine but not by epinephrine (Freestone et al. 2007a). It has been postulated that this specificity reflects the fact that *Y. enterocolitica* infection is principally limited to the gut where epinephrine-containing neurones are not found. This study also showed that epinephrine was a less potent growth inducer for *E. coli* 0157:H7,

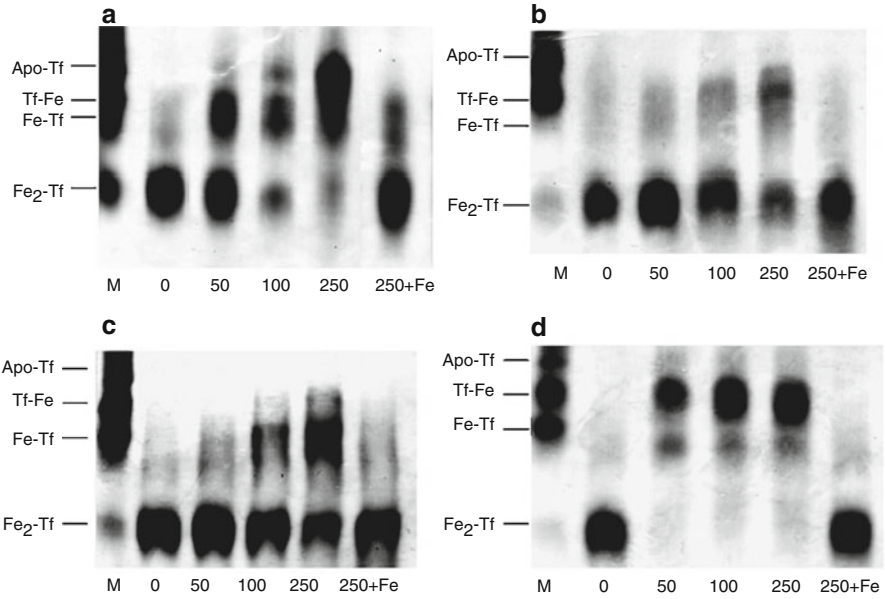

Fig. 4.5 Dietary catechols are able to remove Tf-complexed iron. Urea gels illustrate the effect on the iron-binding status of Tf incubated for 18 h at 37°C in the presence of increasing concentrations of the catechol compounds shown. (**a**) Caffeic acid (lower legend μM); (**b**) catechin (lower legend μM); (**c**) chlorogenic acid (lower legend μM); (**d**) tannic acid (lower legend μg ml^{-1}). *Lane M* contains iron-free (Apo-Tf), monoferric with iron in the N-terminal or C-terminal domains (Fe-Tf and Tf-Fe, respectively), and saturated (Fe_2-Tf) isoforms as markers. +Fe (1 mM ferric nitrate). Reproduced from Freestone et al. (2007c), with permission

with 50 μM epinephrine being required to elicit the stimulation of growth produced by 20 μM norepinephrine or dopamine. Thus, although a general growth response to catecholic substances appears to be common, more specificity may exist than is presently apparent.

In addition to the growth stimulation by neuroendocrine catecholamines, there are also several reports of bacteria utilising these catecholamines as signalling molecules in the activation of expression of colonisation and/or virulence factors. The question therefore arises as to whether these effects are specific to catecholamines or whether similar responses might also be elicited by dietary catechols. Expression of the outer-membrane enterobactin transporter BfeA of *Bordetella bronchiseptica* is activated in response to its substrate by the AraC family transcriptional regulator, BfeR. Anderson and Armstrong (2006) demonstrated that BfeA expression could also be activated by norepinephrine as well as by several other catecholamines and by the non-amine catechols, pyrocatechol and 3,4-dihydroxymandelic acid. It remains to be established whether catechols derived from the diet might also activate BfeA expression.

Although the activation of *B. bronchiseptica* BfeA does not appear to be catecholamine-specific, there is some evidence of catecholamine specificity in *Borrelia burgdorferi*, the causative agent of Lyme disease, in which expression of outer-surface protein A (OspA) is induced by epinephrine and norepinephrine (Scheckelhoff et al. 2007). This induction is blocked by a competitive inhibitor of human β-adrenergic receptors, suggesting that the mechanism is catecholamine-specific. However, since no non-amine catechols were tested, there remains in principle the possibility that other catechols could be bound by, and activate, the as-yet-uncharacterised adrenergic receptor. Importantly, no effect on *B. burgdorferi* growth was seen with either epinephrine or norepinephrine, suggesting that this signalling pathway is independent of iron acquisition.

Additional evidence for the presence of bacterial adrenergic receptors comes from work with *E. coli* 0157:H7. In this bacterium, two response regulators, QseA and QseB, activate virulence gene expression in response to epinephrine, norepinephrine and the bacterial autoinducer AI-3. Binding of epinephrine directly activates QseC, the cognate histidine kinase for QseB, and this activation is blocked by the α-adrenergic antagonist, phentolamine, but not by the β-adrenergic antagonist, propanolol (Clarke et al. 2006). However, both α- and β-adrenergic receptor antagonists block the activation of LEE (Locus of Enterocyte Effacement) gene expression by QseA (Sperandio et al. 2003). This difference in antagonist specificity implies that *E. coli* 0157:H7 possesses an additional, as yet undiscovered, adrenergic-receptor, which is responsible for activating QseA.

Clarke et al. (2006) established that the periplasmic signal-sensing domain of QseC is strongly conserved across a range of bacteria, including *Shigella* sp., *Salmonella* sp., *Erwinia carotovora*, *Haemophilus influenzae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Psychrobacter* sp., and that it shares no primary sequence homology with classical G-protein-coupled adrenergic receptors. It will now be of particular interest to establish the structural features and binding properties of this domain and to discover the extent to which it might also bind, if at all, other catecholic molecules of biological interest. However, the observation that phentolamine antagonizes the effects of norepinephrine upon QseC (Clarke et al. 2006), yet does not block the growth stimulation of *E. coli* 0157-H7 that is seen in response to several dietary catechols when they are provided under iron-restricted conditions (Freestone, unpublished), suggests that growth stimulation by catechols may be mechanistically separate from effects that are mediated via QseC.

The evidence discussed in the last two chapters suggests that catechols in general can stimulate growth of bacteria through their ability to increase the bioavailability of iron. However, studies with inhibitors of adrenergic receptors indicate that there are additional bacterial signalling pathways that are likely to be specific for neuroendocrine catecholamines. There is also evidence that these catecholamines may stimulate growth in a manner independent of iron release from transferrin. It was recently shown in *E. coli* 0157:H7 that adrenergic and dopaminergic receptor antagonists that block growth stimulation by norepinephrine, epinephrine and dopamine do not block ^{55}Fe uptake from ^{55}Fe transferrin (Freestone et al. 2007b). The presence of the

α-adrenergic receptor antagonists did, however, block norepinephrine uptake. However, these effects may still be linked to iron provision since the addition of ferric nitrate overcame the antagonist blockade of growth induction.

4.7 Concluding Remarks

It is apparent that catechols derived from the diet can be present in plasma and in a range of tissues and organs at concentrations that are comparable with those of the neuroendocrine catecholamines and that they both have the potential to promote bacterial growth through the relief of iron restriction. It seems much less probable, on the other hand, that catechols other than catecholamines can mediate responses that require bacterial adrenergic receptors, although definitive experiments have yet to be carried out. However, diet-derived catechols and related compounds may indirectly affect plasma levels of neuroendocrine catecholamines, through the inhibition of catechol-*O*-methyltransferase activity, as demonstrated particularly for (−)-epigallocatechin-3-*O*-gallate (Shixian et al. 2006; Zhu et al. 2008).

Whilst the emphasis in the foregoing discussion has been upon the possible effects on bacteria of dietary catechols in the plasma, catechols and other polyphenols will also influence the behaviour and composition of the colonic microflora. It has been argued that (in plants) a major function of tannins may be to prevent or limit the growth of pathogens and degradative microorganisms, especially (though not exclusively) through the complexation of iron (Scalbert 1991; Mila et al. 1996). It is also well established that a tannin-rich diet has the potential to restrict iron availability in humans (Santos-Buelga and Scalbert 2000; Mennen et al. 2005). It is not certain how far tannins and other catechols present in the gut lumen inhibit bacterial growth in vivo (Chung et al. 1998), or whether in contrast they might, in particular circumstances, *promote* bacterial growth, as shown to occur for *E. coli* O157:H7 and *S. enterica* SV Enteriditis in the serum-SAPI model (Freestone et al. 2007c) and for commensal *E. coli* in an in vivo rat model (Samanta 2004). One factor likely to be important is the low oxygen tension of the gut lumen, which may favour the operation of bacterial iron-uptake mechanisms for Fe^{2+} (Andrews et al. 2003; Naikare et al. 2006). A second factor to be taken into account is the availability and effect of transferrin and lactoferrin, especially given that Freestone et al. (2007c) have demonstrated that dietary catechols can release iron from these glycoproteins and promote its uptake by *E. coli* O157:H7 and *S. enterica* SV Enteriditis. However (in the absence of catechols), both apo-lactoferrin and lactoferrin partially saturated with iron have been found to inhibit the growth of *E. coli* O157:H7 (Griffiths et al. 2003), presumably by chelating any free iron in the media. The growth of the probiotic organism, *Bifidobacterium infantis*, on the other hand, was unaffected. Many lactic-acid bacteria, including *Bifidobacterium* and *Lactobacillus* species, which are widely regarded as beneficial members of the colonic microflora, are notable for their almost complete lack of dependence

upon iron (Pandey et al. 1994; Bruyneel et al. 1989). Therefore, in the gut-lumen environment, these organisms might benefit from factors such as tannins that could inhibit the growth of their iron-requiring competitors.

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Chapter 5
Interactions Between Bacteria and the Gut Mucosa: Do Enteric Neurotransmitters Acting on the Mucosal Epithelium Influence Intestinal Colonization or Infection?

Benedict T. Green and David R. Brown

Abstract The intestinal epithelium is a critical barrier between the internal and external milieux of the mammalian host. Epithelial interactions between these two host environments have been shown to be modulated by several different, cross-communicating cell types residing in the gut mucosa. These include enteric neurons, whose activity is influenced by bacterial pathogens, and their secreted products. Neurotransmitters appear to influence epithelial associations with bacteria in the intestinal lumen. For example, internalization of *Salmonella enterica* and *Escherichia coli* O157:H7 into the Peyer's patch mucosa of the small intestine is altered after the inhibition of neural activity with saxitoxin, a neuronal sodium channel blocker. Catecholamine neurotransmitters, such as dopamine and norepinephrine, also alter bacterial internalization in Peyer's patches. In the large intestine, norepinephrine increases the mucosal adherence of *E. coli*. These neurotransmitter actions are mediated by well-defined catecholamine receptors situated on the basolateral membranes of epithelial cells rather than through direct interactions with luminal bacteria. Investigations of the involvement of neuroepithelial communication in the regulation of interactions between the intestinal mucosa and luminal bacteria will provide novel insights into the mechanisms underlying bacterial colonization and pathogenesis at mucosal surfaces.

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5.1 Introduction

The mechanisms governing the ability of bacteria to adhere to and colonize human and animal hosts in health and disease are still incompletely understood. Throughout the extensive mucosal surfaces of the body that are in contact with the external environment, epithelial cells represent the first point of cellular contact between bacteria and the host. In the intestinal tract, the colonization of the mucosal epithelium by bacteria has become increasingly recognized as an important determinant in the maintenance and protection of health (Ley et al. 2006). Prokaryotic factors, such as flagellin or intimin, play important roles in epithelial adherence or invasion by commensal or pathogenic bacteria; physicochemical factors, such as ambient temperature and pH, contribute to bacterial colonization as well. The roles served by other, host-related factors in microbe–host interactions, such as host regulatory molecules, have recently been discovered. In this chapter, we discuss the nature of intercellular communication that occurs among four key cells, i.e., intestinal epithelial cells (IECs), enteroendocrine cells, neurons, and gut immunocytes, which participate in modulating interactions of bacteria with the intestinal mucosa. We pay special consideration to the emerging role of host-derived biogenic amines in this process. One class of biogenic amines, the catecholamines, epinephrine, norepinephrine (NE) and dopamine (DA) have been extensively studied over the past two decades for their direct effects on the growth and virulence properties of enteric bacteria. This rapidly increasing body of information is discussed elsewhere in this book (cf. Chaps. 3, 6, 9, and 12).

5.2 Gut Bacteria and Conversations Among Cells of the Intestinal Mucosa–Submucosa

A growing body of data indicates that there is extensive intercellular crosstalk among host cells in the intestinal mucosa. Intestinal epithelial cells and enteroendocrine cells, which are situated to first encounter ingested bacteria, chemically communicate with immunocytes and nerves that are located below the mucosal surface. The products secreted from these diverse classes of mucosal and submucosal cells, i.e., cytokines, bioactive peptides, and biogenic amines, affect mucosal defense functions, initiate and regulate inflammatory responses, and alter the outcome of microbe–host associations in health and disease.

5.2.1 Intestinal Epithelial Cells

Intestinal epithelial cells function as accessory immune cells in humans and other mammalian species. They express several types of pathogen-recognition receptors, including Toll-like receptors (TLRs). These receptors are coupled through intracellular

signaling pathways and trigger proinflammatory and host defense responses in IECs. TLRs recognize a variety of pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) and flagellin, which respectively stimulate TLR types 4 and 5. The protective and defensive responses triggered by TLRs and other pathogen-recognition receptors are highly modulated in IECs, probably to permit the coexistence of commensal microflora with the intestinal mucosa. For example, alkaline phosphatase expressed by IECs catalyzes the degradation of LPS, which would otherwise exist at high levels in the intestinal lumen and persistently activate pro-inflammatory signaling cascades in IECs (Bates et al. 2007). Proinflammatory substances, such as interleukin 1-beta or tumor necrosis factor-alpha, act in turn to decrease alkaline phosphatase gene expression in IECs (Malo et al. 2006). In addition, IEC expression of some TLRs appears to be down-regulated, a phenomenon which may serve to limit inflammatory reactions to commensal bacteria contacting these cells (Shibolet and Podolsky 2007). Commensal flora plays an important role in the growth and differentiation of IECs (Hooper 2004).

5.2.2 Enteroendocrine Cells

Mucosal enteroendocrine cells constitute the largest mass of endocrine cells in the body. They are distributed diffusely along the length of the intestine where they are poised to sample luminal contents and come into contact with mucosa-associated bacteria. Compared to animals with normal gut microbiota, germ-free animals manifest differences in the numbers and hormonal contents of enteroendocrine cells (Uribe et al. 1994; Sharma and Schumacher 1996). Recent evidence indicates that some enteroendocrine cells express functional taste and olfactory receptors (Braun et al. 2007; Sternini et al. 2008) as well as TLRs (Bogunovic et al. 2007; Palazzo et al. 2007). The secreted products of enteroendocrine cells, including serotonin and a diverse array of gut peptide hormones, act directly and through neurons and most likely gut leukocytes to control aspects of epithelial secretion, growth, and defense.

5.2.3 Enteric Neurons

Serotonin and gut peptide hormones released from enteroendocrine cells interact with nerves and leukocytes in the intestinal submucosa as well. There are in excess of 100 million intrinsic neurons surrounded by supporting glial cells that reside within the intestinal wall (Fig. 5.1). In addition, neurons lying outside the intestine send projections to target cells in the intestinal wall and mucosa. Neurons projecting to the mucosa participate in the monitoring of intestinal contents and regulation of mucosal defense function; their activity is modulated by mediators derived from IECs, enteroendocrine cells, and immunocytes (Levite and Chowers 2001;

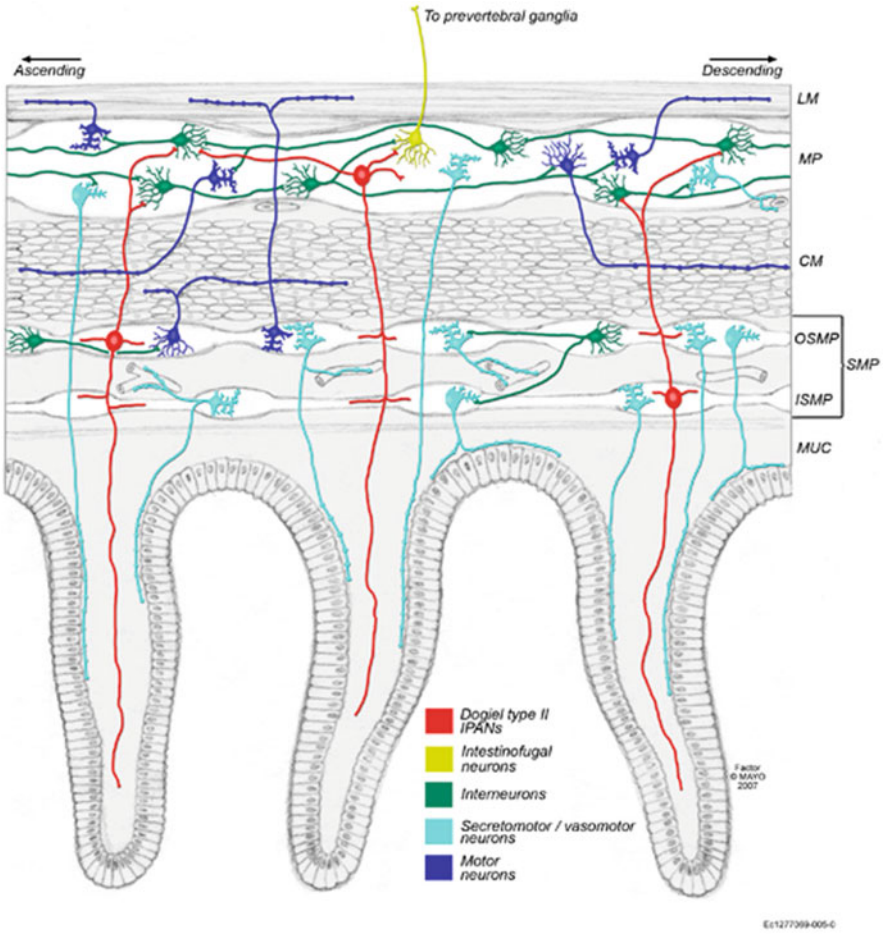

Fig. 5.1 Functional classes of enteric neurons and their major projections to the mucosa, submucosal neurons, and smooth muscle coats in the intestinal wall of a large mammal, such as a pig. Note that the mucosa receives both afferent and efferent innervation. *CM* circular muscle; *IPAN* intrinsic primary afferent neuron; *ISMP* and *OSMP* inner and outer submucosal plexuses; *LM* longitudinal muscle; *MP* myenteric plexus; *MUC* mucosa (from Linden and Farrugia 2008)

Downing and Miyan 2000; Lundgren 2004). Like these other cell types, enteric neurons appear to express TLRs capable of detecting bacteria-associated molecular patterns (Barajon et al. 2009; Rumio et al. 2006; Arciszewski et al. 2005). There is also evidence that commensal flora is necessary for proper enteric nervous system (ENS) structure and function (Dupont et al. 1965).

5.2.4 *Diffuse and Organized Gut-Associated Lymphoid Tissue*

The gut-associated lymphoid tissue (GALT) represents the largest component of the common mucosal immune system and functions to control intestinal infections. It consists of a diffuse lymphoid compartment containing large populations of lymphocytes and antigen-presenting cells (e.g., macrophages, dendritic cells) in the intestinal lamina propria and Peyer's patches, which are organized lymphoid follicles covered by a single layer of specialized epithelial cells (i.e., M cells). Studies of germ-free and gnotobiotic rodents have shown that microflora play a role in the development of the GALT (Bauer et al. 2006). As the inductive site for mucosal immunity, Peyer's patches play a critical role in sampling of luminal contents and initiating adaptive immune responses towards potentially harmful microorganisms and antigenic materials (Mowat 2003). This includes the generation of immunoglobulin A (IgA)-producing lymphoblasts. These cells mature in the system circulation and then traffic as plasma cells to mucosal effector sites in the gut lamina propria. Neurons and nerve fibers exist in close proximity to lamina propria leukocytes, including mast cells (Wood 2007) and lymphocytes (Downing and Miyan 2000). Peyer's patches are highly innervated (Defaweux et al. 2005; Vulchanova et al. 2007; Chiocchetti et al. 2008). Antigen-specific secretory IgA synthesized in these plasma cells is the major immunoglobulin secreted onto mucosal surfaces and plays an important role in mucosal protection; furthermore, constitutively produced secretory IgA is thought to regulate the gut microfloral population (Suzuki et al. 2007; Macpherson and Slack 2007). In addition to their important immunological role, Peyer's patches are exploited as portals of entry into the body for several species of enteropathogenic bacteria (Clark and Jepson 2003).

5.3 The Enteric Nervous System, Mucosally Directed Nerves, and Gut Bacteria

5.3.1 *Organization of the Enteric Nervous System*

The mammalian ENS originates mainly from the vagal neural crest during gestation and constitutes two or more distinct ganglionated plexuses (Burns and Thapar 2006). These include the submucosal (Meissner) and myenteric (Auerbach) plexuses, which are organized into an interconnected neural network of reflex arcs containing intrinsic primary afferent neurons, interneurons, and excitatory or inhibitory, motor and secretomotor neurons, each with distinct plurichemical coding (Furness 2006). Thus, the intestine is the only visceral organ capable of executing complex preprogrammed behaviors that can occur independently of the central nervous system.

The myenteric plexus, which is located between the longitudinal and circular smooth muscle layers, coordinates intestinal propulsion and segmentation. It is well known that neurally mediated disruptions in myoelectrical activity and mechanical functions of the intestine can alter the enteric content of microorganisms (Scott and Cahall 1982).

Neurons in the submucosal plexus(es) regulate active ion transport and paracellular permeability of the intestinal epithelium, as well as relay sensory information from the mucosa to the myenteric plexus and central nervous system (Furness 2006). Species differences have been observed in the structure and chemical coding of submucosal neurons. Rodents possess a single submucosal ganglionated plexus whereas larger mammals such as cattle and pigs have both inner (Meissner's) and outer submucosal (Schabadasch) plexuses (Fig. 5.1). Humans possess a third submucosal plexus, which appears to be similar in neurochemical coding to the outer submucosal plexus (Timmermans et al. 2001).

5.3.2 The ENS and Gut Bacteria

Submucosal and myenteric nerves play an important defensive role in the initial stages of bacterial infection, by coordinating the intestinal secretory and propulsive functions necessary to dilute and purge enteropathogens (Spiller 2002). Ingested pathogenic bacteria can alter enteric neural activity and plasticity, either through (1) direct interactions with nerve cell bodies and fibers, or (2) induction of inflammatory responses in neurons and neighboring cells, such as enteric glia, or (3) the release of neuroactive exotoxins (Lundgren 2002). Enteric neuropeptides mediate or mitigate these bacterial effects on the ENS. Some gut neuropeptides, including SP, neuropeptide Y, and neurotensin, even possess inherent antimicrobial activity (Brogden et al. 2005).

LPS at high concentrations (100 ng/ml) produces death in cultured myenteric neurons, an effect that can be prevented by the enteric neurotransmitter vasoactive intestinal peptide (VIP) (Arciszewski et al. 2005, 2008). *Bacteroides fragilis* infections alter the relative proportions of substance P- and somatostatin-expressing colonic neurons and fibers (Gonkowski et al. 2003). Intestinal infection with the causative agent in porcine proliferative enteropathy, *Lawsonia intercellularis*, is associated with an increase in the number of submucosal neurons immunoreactive for the gut peptides VIP, galanin, somatostatin, and calcitonin gene-related peptide (Pidsudko et al. 2008). The probiotic bacterium Lactobacillus reuteri appears to modulate enteric neurotransmission linked to colonic motility by affecting the gating properties of neuronal cation channels (Wang et al. 2010). Several disease-causing bacteria, including enteropathogenic *Escherichia coli*, *Salmonella Typhimurium*, and *Shigella dysenteriae*, induce the expression of receptors for the enteric neuropeptide galanin on colonic epithelial cells; this effect is not produced by normal colonic microflora. Galanin released by colonic submucosal nerves acts upon these epithelial cell receptors

to stimulate active transepithelial anion secretion, which in turn contributes to secretory diarrhea (Hecht et al. 1999; Matkowskyj et al. 2000). The gut neuropeptide substance P (SP), which activates intestinal defenses against bacteria, has been shown to reduce host susceptibility to enteric *Salmonella* infections (Pascual 2004). This phenomenon appears to be due to the involvement of this neuropeptide and its cognate receptors in Salmonella-induced gut inflammation (Walters et al. 2005).

Toxins from *Clostridium difficile*, *Shigella* spp., *Campylobacter* spp., and other enteropathogens can evoke the release of inflammatory mediators, which can acutely alter neuronal activity and chronically produce structural and chemical changes in the ENS (Vasina et al. 2006; Lomax et al. 2006). The copious diarrhea associated with *Vibrio cholerae* infections has been attributed in part to cholera toxin-evoked increases in the activity of enteric neurons expressing VIP, a potent secretogogue (Mourad and Nassar 2000). *Clostridium difficile* toxin B appears to activate VIPergic submucosal neurons via an interleukin 1-dependent mechanism (Neunlist et al. 2003). Fluid secretion in diarrhea associated with *Salmonella enterica* and enterotoxigenic *E. coli* infections may involve enteric neural circuits as well (Lundgren 2002).

5.3.3 Catecholamines in the ENS

Both DA and NE are synthesized in and released from enteric nerves innervating the intestinal mucosa (Wu and Gaginella 1981; Llewellyn-Smith et al. 1981, 1984; Eisenhofer et al. 1997; Vieira-Coelho and Soares-da-Silva 1993; Wang et al. 1997; Li et al. 2004; Lomax et al. 2010). Enteric dopaminergic neurons appear to reside within the intestinal wall, but the noradrenergic innervation of the intestine originates in neurons lying outside the gut wall are located in prevertebral ganglia (Anlauf et al. 2003; Li et al. 2004; Furness 2006). These "extrinsic" sympathetic nerve fibers may co-contain peptide neurotransmitters such as neuropeptide Y or somatostatin (Timmermans et al. 1997; Straub et al. 2006, 2008). Butyrate, which is produced by colonic bacteria, has been found to transcriptionally activate the gene encoding tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis (Patel et al. 2005). Epinephrine is not synthesized in enteric nerves because phenylethanolamine *N*-methyltransferase, the enzyme catalyzing epinephrine synthesis from norepinephrine, does not appear to be expressed in the digestive tract (Black et al. 1981; Bäck et al. 1995; Kennedy and Ziegler 2000; Costa et al. 2000). Interestingly, the growth of enteropathogens such as enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) and *Salmonella enterica* is preferentially enhanced by catecholamines that are normally present in the GI tract (NE and DA) in comparison to epinephrine, and growth of the more exclusive enteric pathogen *Yersinia enterocolitica* is stimulated by NE and DA but not epinephrine (Freestone et al. 2007a). The direct action of NE on bacterial growth is unlikely to be mediated by conventional adrenergic receptors, which recognize both NE and epinephrine (cf. Sect. 5.3.4 below).

In addition to neuronal cells, there is accumulating evidence that immune cells are capable of synthesizing, releasing, and degrading catecholamines. As there is an abundance of immunocytes within the GALT, these cells may represent an alternate, non-neuronal source of NE and DA in the intestinal wall (Flierl et al. 2008).

5.3.4 Catecholamine Receptor Pharmacology

Norepinephrine and DA activate their cognate G protein-coupled receptors expressed on closely apposed neurons, IECs, and other target cells, which influence overall mucosal function and alter intestinal susceptibility to infection. Receptors for NE and DA have been defined over approximately four decades through the development and use of highly selective receptor agonists and antagonists in functional pharmacological investigations and, more recently, through molecular cloning and structure-function studies in isolated cells and transgenic animals.

The receptor concept was based at the turn of the last century on the powerful and physiologically relevant approach of defining binding interactions of endogenous substances or their synthetic homologs with specific receptors that are linked to a biological response. Biochemical analyses of selective ligand-binding site interactions that were developed some 60 years later provide valuable information on the affinities (K_d, dissociation constant) and competitive interactions of ligands at specific binding sites as well as the relative density (B_{max}) of the binding sites. Because they do not measure the biological activity of the ligands examined, however, they do not truly define a "receptor," which is an entity that is functionally coupled to intracellular signal transduction pathways and mediates a biological function. Binding studies, if carefully executed, provide information on a specific binding site for an endogenous or synthetic ligand, which can serve as supporting evidence for the presence of the receptor in a biological system. Through GTPγ^{35}S binding assays (Harrison and Traynor 2003), it is now possible to assess the effectiveness of ligands to activate G proteins coupled to a particular receptor and this approach affords a better approximation of drug activity (e.g., it is possible to distinguish an agonist from an antagonist). There is generally a good concordance in the results of studies determining the affinities and competitive interactions among ligands for adrenergic receptors (ARs) and dopaminergic receptors (DRs) by functional means with affinities and interactions of the same ligands determined biochemically at specific binding sites.

Presently, nine ARs are classified into alpha- and beta-AR types; there are two subtypes of alpha-ARs ($alpha_1$ and $alpha_2$) having three isoforms each, and three subtypes of beta-ARs. Compared to epinephrine, NE has relatively higher binding affinity for alpha-ARs and $beta_1$- and $beta_3$-ARs, but lower affinity for $beta_2$-ARs. Two main DR types exist through gene duplication events in the vertebrate lineage (D_1R and D_2R); from these, five receptor subtypes have been defined. Our understanding of the nature of these receptors and their relationships to G proteins and downstream intracellular signaling cascades is evolving (Strange 2008).

For example, there is accumulating evidence that these receptors may form homo- or heterodimeric complexes on cell membranes that possess a pharmacological profile that differs from that of the monomeric receptor(s) or may be involved in the process of receptor down-regulation. With respect to ARs, this dimerization phenomenon appears to occur between different ARs as well between ARs and other classes of G protein-coupled receptors, including chemokine receptors (Milligan et al. 2005; Hague et al. 2006); dopamine receptors are known to form heterodimeric complexes with adenosine receptors (Fuxe et al. 2005).

5.3.5 *Catecholamine Receptors and Mucosal Function*

Enteric dopaminergic and alpha$_1$-, alpha$_2$-, or beta-adrenergic binding sites have been detected on submucosal nerves or IECs in some species, notably the guinea pig and rat (Chang et al. 1983; Cotterell et al. 1984; Senard et al. 1990; Valet et al. 1993; Vieira-Coelho and Soares-da-Silva 2001; Baglole et al. 2005). Catecholamine receptors are probably expressed by immunocytes and enteroendocrine cells as well. NE alters immunocyte function (Elenkov et al. 2000; Meredith et al. 2005; Kin and Sanders 2006) and modulates serotonin release from enterochromaffin cells in the intestinal mucosa (Pettersson 1979; Simon and Ternaux 1990; Schäfermeyer et al. 2004). In summary, enteric catecholamines, NE in particular, can potentially modulate crosstalk between several different types of cells in the intestinal mucosa and submucosa.

Norepinephrine alters active, transepithelial ion transport in the intestinal mucosa through interactions with functionally defined alpha-ARs and to a lesser extent the beta-ARs. Depending upon the animal species and intestinal segment examined, this action is mediated indirectly through enteric nerves or by direct effects on IECs (Brown and O'Grady, 1997; Horger et al. 1998). In addition, NE modulates epithelial growth and turnover (Tutton and Helme 1974; Tutton and Barkla 1977; Olsen et al. 1985), paracellular permeability (Lange and Delbro 1995), and the vectorial secretion of secretory IgA towards the gut lumen (Schmidt et al. 1999, 2007). Dopamine affects active ion transport through direct and indirect actions on enteric adrenergic and dopaminergic receptors (Donowitz et al. 1982, 1983; Vieira-Coelho and Soares-da-Silva 1998; Al-Jahmany et al. 2004).

5.3.6 *Enteric Nerves, Catecholamines, and IEC: Bacteria Interactions*

Both NE and DA have been shown to alter the mucosal attachment or invasiveness of bacterial pathogens such as EHEC or serovars of *Salmonella enterica* not always through direct contact with these bacteria, but rather by acting on cells of the intestinal

Table 5.1 Functional evidence for mucosal *alpha*-adrenergic receptors influencing EHEC adherence to explants of porcine cecal and colonic mucosae

Pharmacological characteristic	Supporting evidence	References
Selective agonism	At equimolar concentrations, UK14,304 (alpha$_2$-adrenoceptor agonist), but neither phenylephrine (alpha$_1$) nor isoproterenol (beta) increase EHEC adherence to cecal mucosa	Chen et al. (2006)
Selective antagonism	At equimolar concentrations in both cecum or colon and phentolamine (alpha-adrenoceptor antagonist), but not propranolol (beta), inhibits NE action. Furthermore, yohimbine (alpha$_2$), but not prazosin (alpha$_1$), inhibits NE action	Green et al. (2004) and Chen et al. (2006)
Laterality of drug action	NE at low (μM) concentrations effective only when added to the contraluminal, but not luminal bathing medium, consistent with submucosal localization of adrenergic receptors	Green et al. (2004)

mucosa (Table 5.1). The actions of these catecholamines on bacteria–mucosa interactions have been examined in mucosal explants mounted in Ussing chambers (Brown and O'Grady 2008). This apparatus has been used for decades in studies of transepithelial ion transport, and more recently in investigations of bacteria–host interactions (Ding et al. 2001; Crane et al. 2006). This system extends the viability of mucosal explants under quasi-physiological conditions, allows for continuous, tangential flow of bacteria across a fixed mucosal surface area, and permits the selective contact of drugs and bacteria with the luminal or contraluminal surfaces of intestinal tissues (Fig. 5.2).

5.3.6.1 Role of the ENS and Catecholamines in Bacterial Internalization into the Mucosa of the Small Intestine

The attachment and invasion of enteropathogenic bacteria to the intestinal mucosa appears to be modulated by the ENS. Inhibition of neural conduction by the serosal side addition of the neuronal sodium channel blocker saxitoxin increases internalization of luminally inoculated *Salmonella enterica* serovar Choleraesuis and EHEC by >6-fold in Peyer's patch explants from the porcine jejunum. Internalization of a rodent commensal *E. coli* strain is unaffected by the toxin (Green et al. 2003) and that of *S. enterica* serovar Typhimurium is decreased by threefold (Brown and Price 2008). Serosal application of the neurotoxin or the local anesthetic lidocaine decreased *S. Typhimurium* internalization by three- to fourfold in explants of non-Peyer's patch absorptive mucosa from porcine jejunum. In contrast, electrical stimulation of enteric nerves in this preparation increased *S. Typhimurium* internalization by 2.5-fold and this effect was inhibited by saxitoxin or lidocaine

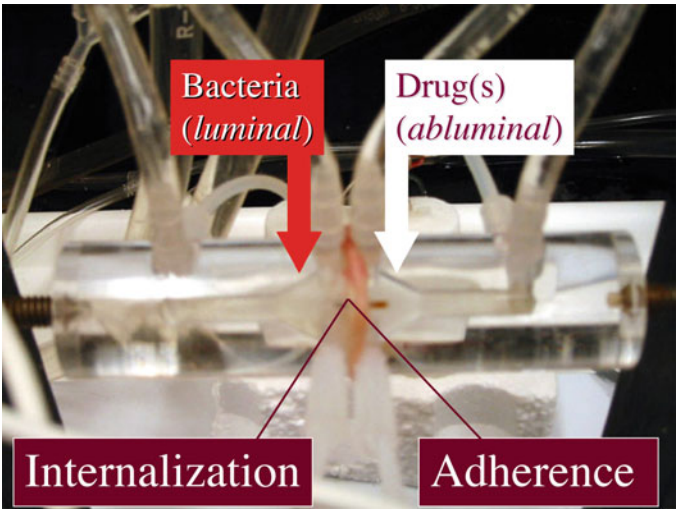

Fig. 5.2 Photograph of an Ussing chamber containing a porcine intestinal mucosa explant. *Arrows* indicate the locations of bacterial inoculations and drug additions as performed in many experiments such as those summarized in Table 5.1

(Schreiber et al. 2007). Although these neurally mediated effects on *Salmonella* internalization may appear to be small, it should be emphasized that they were measured over a surface area of 2 cm^2 in isolated tissues over a relatively short (90 min) time period. If extrapolated to the large surface area encompassed by the small intestine or even a segment thereof, these changes in Salmonella uptake are likely to be biomedically significant.

The cellular mechanisms underlying these neurally mediated effects on Salmonella internalization in the porcine small intestinal mucosa are undefined at present. There is evidence that they differ for different serovars Choleraesuis and Typhimurium of *S. enterica* as saxitoxin increases internalization of the former and decreases that of the latter. Moreover, Salmonella internalization is inhibited by the actin polymerization inhibitor cytochalasin D in the nonfollicular absorptive mucosa and monolayers of the porcine enterocyte cell line, IPEC J2, a result that is in agreement with other studies of actin-dependent Salmonella invasion in epithelial preparations (Schreiber et al. 2007; Brown and Price 2007). In contrast, cytochalasin D has no effect on the uptake of *S. enterica* serovars Cholerasuis and Typhimurium into jejunal Peyer's patch mucosa explants (Green and Brown 2006; Brown and Price 2008).

Norepinephrine and DA have also been implicated in *Salmonella* internalization, especially in porcine jejunal Peyer's patches, where there is strong immunohistochemical evidence for catecholaminergic innervation (Kulkarni-Narla et al. 1999) At these inductive sites for mucosal immunity, nerve fibers immunoreactive for the catecholamine synthetic enzymes tyrosine hydroxylase and dopamine beta-hydroxylase can be seen terminating beneath epithelial cells. Enteric nerves near

Peyer's patch follicles express immunoreactivities for the type 2 vesicular monoamine transporter, which transports catecholamines into synaptic vesicles, and the norepinephrine transporter NET, a target of cocaine action (Kulkarni-Narla et al. 1999; Green et al. 2003).

The serosal application of NE at a bath concentration of 10 μM produced a six- to ninefold increase in luminal *S. enterica* serovar Choleraesuis and EHEC internalization in porcine Peyer's patch explants. This effect was not mimicked by luminally-applied NE, but was inhibited in tissues pretreated with the alpha-AR antagonist phentolamine. These results indicate that this NE action is mediated by alpha-ARs which are likely localized to the basolateral aspect of Peyer's patch epithelial cells (Green et al. 2003). In a study of *S. enterica* serovar Typhimurium internalization, the serosal administration of DA or the sympathomimetic drugs cocaine and methamphetamine decreased Salmonella recovery from Peyer's patch explants (Brown and Price 2008). It is not known if these effects of NE and DA extend to species other than swine. Although the underlying cellular mechanisms for them must be investigated further, it is tempting to hypothesize that catecholamines may regulate the sampling function of Peyer's patches to control the entry or immune processing of pathogenic microbes at these intestinal sites.

5.3.6.2 Catecholamines and EHEC Adherence to the Mucosa of the Large Intestine

When added to the medium bathing the contraluminal surface of cecal explants from mice, NE and DA increase the number of EHEC adhering to the mucosal surface. They do so at 50% effective concentrations (EC_{50}) of 3.8 and 4.2 μM, respectively. The concentrations of NE applied to the basolateral aspect of the intestinal epithelium that are sufficient to promote EHEC adherence are somewhat lower than those necessary to promote epithelial EHEC adherence when incubated directly with the bacterium (Vlisidou et al. 2004; Bansal et al. 2007). The adherence-enhancing actions of NE and DA on the epithelium are inhibited respectively by AR and DR receptor antagonists, a result indicating that they are mediated by specific catecholamine receptors (Chen et al. 2003). This appears to differ from the mechanism by which these catecholamines produce their direct effects on bacterial function (Freestone et al. 2007b).

This phenomenon extends to species other than the mouse. Indeed, in mucosal explants of porcine cecum and colon, NE increases mucosal adherence of EHEC through interactions with $alpha_2$-ARs that were characterized by conventional receptor criteria (Table 5.1). Increases in active anion secretion across the porcine colonic mucosa are in comparison mediated by $alpha_1$-ARs (Brown and O'Grady 1997). Therefore, it appears that the actions of NE on ion transport and EHEC adherence are not linked through a common cellular mechanism. A$lpha_2$-ARs are negatively coupled to cyclic AMP production and a concomitant decrease in intracellular protein kinase A activity. In support of this receptor–effector association, the adherence-promoting action of NE in the porcine colonic mucosa is inhibited

by the protein kinase A activator Sp-cAMPS and mimicked by the protein kinase A inhibitor Rp-cAMPs (Green et al. 2004). The effects of NE in the mouse and pig cecal mucosae are relatively rapid (≤90 min), and experiments with EHEC eae and EspA deletion mutants strongly suggest that NE and other sympathomimetic drugs enhance early, nonintimate bacterial adherence (Chen et al. 2003, 2006). As with their effects on *Salmonella* internalization, although the effects of NE and other sympathomimetic drugs on EHEC adherence may appear small (<1.0 log unit increase in the number of adherent bacteria in mucosal explants with an exposed surface area of 1 or 2 cm^2), they may assume considerable medical importance when extrapolated over the extensive surface area of the cecal or colonic mucosa (Snipes 1997).

The mechanisms underlying this unique catecholamine action remain to be further defined through the identification of epithelial surface factors that mediate bacterial adherence and the receptor–effector pathways that are linked to their rapid expression. Beta1-integrins are IEC surface receptors implicated in aspects of EHEC adherence (Sinclair et al. 2006). By blocking epithelial beta1-integrins, heparin has been shown to inhibit EHEC adherence to human colonic epithelial cells (Gu et al. 2008). Norepinephrine is known to enhance interactions between blood cells and the vascular endothelium by stimulating the rapid expression of beta1-integrins (Levite et al. 2001; Butta et al. 2004; Delahunty et al. 2006), and it is tempting to speculate that it may similarly do so in promoting IEC interactions with luminal bacteria. In addition to dissecting the cellular and molecular mechanisms underlying this phenomenon, studies of catecholamine action on bacterial adherence in vitro should be extended to investigations of the role of endogenous and exogenous DA and NE in isolated intestinal loops and intact animal models which encompass larger surface areas and have greater translational relevance.

Norepinephrine may play a physiological role in promoting bacterial colonization of the large intestine, perhaps as an element in host defense. This hypothesis is based in part on a finding that NE increases cecal adherence of a non-O157 strain of *E. coli*, which was isolated from the porcine colonic mucosa (Chen et al. 2006). One interpretation of this result is that the action of NE is not limited to a particular bacterial strain or species. Presumptive NE nerve fibers immunoreactive for dopamine *beta*-hydroxylase are present throughout the submucosa and appear to terminate near the basal membranes of crypt and surface epithelial cells of the porcine distal colon and cecum (Green et al. 2004; Chen et al. 2006). Drugs capable of inhibiting the degradation (such as the monoamine oxidase inhibitor, pargyline) and neural reuptake (desipramine, cocaine) of NE at neuroepithelial junctions mimic the EHEC adherence-promoting action of NE, and their effects are inhibited by phentolamine (Green et al. 2004; Chen et al. 2006). In the porcine colonic mucosa, dopamine beta-hydroxylase-immunoreactive nerves terminate near IgA-positive B lymphocytes and neighboring IECs immunoreactive for the polymeric immunoglobulin receptor (Schmidt et al. 2007). Norepinephrine stimulates the vectorial secretion of secretory IgA in porcine colonic mucosa explants. This effect has been attributed to an alpha-AR-mediated increase in the luminally directed transport of secretory factor, a component of the polymeric Ig receptor (Schmidt et al. 2007).

As noted above, constitutively produced secretory IgA is hypothesized to modulate colonization of the intestinal mucosa by commensal bacteria (Suzuki et al. 2007; Macpherson and Slack 2007).

5.4 Other Biogenic Amines and Gut Bacteria

Histamine is another biogenic amine transmitter that has been implicated in intestinal host–pathogen interactions. Its synthesis from the amino acid histidine is catalyzed by histidine decarboxylase. It has long been known that the expression of the inducible isoform of this enzyme is increased by LPS (Oh et al. 1988). Indeed, ingestion of LPS increases the histamine content of the intestinal tract (Aschenbach et al. 2003). Histamine generated by histidine decarboxylase induced by *E. coli* acts via H_1- and H_2-histamine receptors to reduce the clearance of *E. coli* from the peritoneal cavity in a murine experimental peritonitis model (Hori et al. 2002). Selective agonists at each receptor mimicked the effects of *E. coli* and exogenous histamine; on the other hand, the selective H_1- and H_2-histamine receptor antagonists pyrilamine and cimetidine, respectively, accelerated the peritoneal clearance of this microorganism. *Yersinia enterocolitica* infection of murine Peyer's patches is similarly associated with the induction of histidine decarboxylase and consequent generation of histamine. For example, cimetidine, but not pyrilamine, decreased the survival of *Yersinia*-infected mice, an effect that may be related to a suppression of H_2-histamine receptor-induced innate immune responses to this pathogenic bacterium (Handley et al. 2006).

The indoleamine serotonin (5-hydroxytryptamine) is released from enteric neurons and mucosal enterochromaffin cells. Although the gut contains most of the body's serotonin content, little is known of the potential effects of this biogenic amine on microbial interactions with the intestinal mucosa. Infections produced by *Citrobacter rodentium*, a Gram-negative bacterial pathogen that adheres to the colonic epithelium of mice, are associated with an increase in the stimulated release of serotonin from the colonic mucosa, but reduced numbers of enterochromaffin cells exhibiting immunoreactivity for serotonin and its uptake transporter (O'Hara et al. 2006). Enteropathogenic E. coli also appears to inhibit the serotonin transporter (Esmalli et al. 2009). These changes in mucosal serotonin signaling appear to be a consequence of mucosal immune responses to this pathogen. Indeed, there is recent evidence that enterochromaffin cell number and serotonin content may be influenced by the types of cytokines released in the course of enteric infections (Wang et al. 2007; Motomura et al. 2008).

5.5 Conclusions

The potential involvement of biogenic amines in intestinal interactions with bacteria and their connection to organismal biology is of emerging interest. The biological significance of the effects of the catecholamines, histamine, and serotonin on

mucosal interactions with bacteria remains a mystery. Despite the roles of the ENS and the diverse array of neurotransmitters it contains in responding to bacterial infection, their involvement in host–pathogen interactions at the cellular level has yet to be firmly established.

In addition to their direct effects on bacterial growth and virulence, catecholamines released from enteric nerves appear to selectively act upon host receptors to alter mucosal internalization or adherence of intestinal bacteria. Their actions are exerted at mucosal inductive and effector sites in the intestinal tract and underscore the complex interactions of these neurotransmitter substances with IECs, enteroendocrine cells, neurons, and immunocytes in the intestinal mucosa and submucosa. Indeed, NE and possibly DA may serve roles in the regulation of bacterial sampling at immune recognition and processing sites and in the establishment or maintenance of mucosa-associated microfloral populations. The role of neuropeptides including neuropeptide Y and somatostatin or other substances (e.g., adenosine 5′-triphosphate) coreleased with NE from enteric sympathetic nerve terminals remains unknown, but they may serve to modulate NE actions. Neuropeptide Y and ATP, for example, have been shown to modulate the vasomotor effects of NE on blood vessels receiving adrenergic innervation (Huidobro-Toro and Donoso 2004).

With particular reference to NE, it is tempting to speculate that stress-induced sympathetic neural outflow may influence mucosal interactions with intestinal bacteria. This notion is supported by the finding that another stress-evoked neuroactive substance, adrenocorticotrophic hormone, acts like NE to enhance the colonic adherence of EHEC (Schreiber and Brown 2005). Sympathetic nerves are also implicated in intestinal inflammatory states, and the relationships of noradrenergic activity with intestinal inflammation, colonization of mucosa-associated commensal bacteria, and predisposition to enteric infections offers a fruitful area for future investigations (Irving and Gibson 2008; Lawley et al. 2008; Straub et al. 2008).

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Chapter 6
Modulation of the Interaction of Enteric Bacteria with Intestinal Mucosa by Stress-Related Catecholamines

Mark P. Stevens

Abstract Stress associated with parturition, transport or mixing has long been correlated with enhanced faecal excretion of diarrhoeal zoonotic pathogens in animals such as *Salmonella enterica* and *Escherichia coli*. It may also predispose humans to infection and/or be associated with more severe outcomes. One possible explanation for this phenomenon is the ability of enteric bacterial pathogens to sense and respond to host stress-related catecholamines. This article reviews evidence of the ability of catecholamine hormones to modulate interactions between Gram-negative diarrhoeal pathogens and intestinal mucosa, as well as the molecular mechanisms that may be at work.

6.1 Introduction

Stress and susceptibility to microbial infection have long been correlated. A plausible explanation for this link is that persistent activation of adrenal axes under chronic stress or depression leads to the release of soluble mediators that may impair innate and adaptive immunity (reviewed in Nance and Sanders 2007). Indeed, neurotransmitters, neuropeptides and adrenal hormones modulate specific and nonspecific activities of the cell-mediated immune response by binding to cellular receptors and sympathetic nerve fibres, densely innervate lymphoid organs and terminate in the proximity of immune cell populations. In recent years however, it has become clear that many bacteria are able to sense mediators of the host stress response and respond by activating growth and the expression of virulence factors. Particular emphasis has been placed on the ability of Gram-negative enteric pathogens to respond to stress-related catecholamine hormones. This article reviews evidence that stress is correlated to the outcome of enteric bacterial infections and examines the molecular mechanisms that may be at work.

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6.1.1 Stress and Enteric Bacterial Infections in Animals

In food-producing animals, stress associated with social interaction, handling and transport has been correlated with increased excretion of pathotypes of *Escherichia coli* and *Salmonella enterica*. An understanding of the dialogue between such pathogens and the stressed host is important, not only in the interests of animal welfare, but because the organisms may transmit through the food chain and environment to humans, where they may cause acute enteritis and severe systemic sequelae. Studies in animals may also inform us of events relevant in the pathogenesis of human bacterial diseases and aid the development of strategies to interfere with host-pathogen signalling.

In young piglets, isolation from the sow, short-lived cold stress or mixing of litters has been reported to increase faecal excretion of enterotoxigenic *E. coli* (ETEC) relative to control piglets (Jones et al. 2001). Measurement of antibody and T-cell proliferation against a model antigen (ovalbumin) administered intramuscularly at the time of oral ETEC challenge revealed no differences in the immune response between control and stressed piglets, indicating that enhanced faecal excretion of ETEC under stress may not involve modulation of host adaptive immunity. Even mild physical handling of pigs, involving a daily weight measurement requiring movement to-and-from scales in a proximal pen, increased the faecal excretion of *E. coli* and total coliforms relative to control pigs (Dowd et al. 2007). Conclusive data that transport or social stress influence the carriage and virulence of other *E. coli* pathotypes is lacking, however, elevated growth of enterohemorrhagic *E. coli* (EHEC) serotype O157:H7 could be detected in semi-permeable chambers implanted in the peritoneal cavity of mice in a social conflict model, relative to control nonstressed mice (Dréau et al. 1999).

In the case of *Salmonella*, transportation has been correlated with reactivation of subacute *S. enterica* serovar Typhimurium infections in pigs (Isaacson et al. 1999), and with increased hide and faecal *Salmonella* contamination in transported beef cattle (Barham et al. 2002). Social stress caused by mixing has also been found to increase faecal excretion and invasion of *S.* Typhimurium in early-weaned pigs (Callaway et al. 2006). Mixed pigs also exhibited elevated cecal coliform counts and increased translocation of *Salmonella* to intestinal lymph nodes (Callaway et al. 2006). Although studies in poultry are lacking, evidence is emerging that stress associated with stocking density, access to feed and water, physical disturbance and depopulation (thinning) may be correlated with the carriage of zoonotic pathogens and disease susceptibility (reviewed in Humphrey 2006).

6.1.2 Response of the Enteric Nervous System to Stress and Implications for Enteric Bacteria

A key response of the enteric nervous system (ENS) to stress is the release of the catecholamine hormones. Catecholamines are derived from tyrosine and possess a benzene ring with adjacent hydroxyl moieties and an opposing amine side chain.

Norepinephrine (NE) is released from sympathetic nerve fibres originating in the prevertebral ganglia that innervate the gut mucosa (reviewed in Furness 2006). In contrast, dopamine (DA) is expressed in a subpopulation of nonsympathetic enteric neurons residing in the intestinal wall. It has been estimated that the human ENS may comprise at least 500 million neurons (Furness 2006). Nerves reactive to antibody against tyrosine hydroxylase (which catalyses the rate-limiting step in NE synthesis), alone or in combination with dopamine β-hydroxylase (which mediates the terminal step in NE synthesis), have been found to innervate porcine intestinal mucosa with some terminating close to the lumen (Fig. 6.1; Kulkarni-Narla et al. 1999; Green et al. 2003). Neurons containing phenylethanolamine *N*-methyltransferase, which is required for the synthesis of epinephrine from NE, are lacking in the intestinal mucosa (Costa et al. 2000), and it is therefore considered unlikely that epinephrine would be found in significant quantities in the gut, except perhaps when the mucosal barrier is substantially damaged.

Significant spillover of catecholamines from the systemic circulation into the intestines can occur during episodes of acute stress (Aneman et al. 1996; Eisenhofer et al. 1997) and enhanced release of catecholamines by the ENS under stress has been demonstrated experimentally. Intestinal expression of tyrosine hydroxylase is upregulated in response to surgical perforation of the bowel and gut-derived sepsis in rats (Zhou et al. 2004) and catabolic stress induced by partial hepatectomy in mice has been reported to result in elevated levels of NE in faecal pellets compared to those from mice subject to sham-laparotomy (Alverdy et al. 2000). Evidence that such events may be significant in the progression of enteric disease was provided by the finding that NE and DA are able to induce the growth of commensal *E. coli* in vitro (Freestone et al. 2002). It was subsequently shown that NE stimulates the growth of Gram-negative enteric pathogens of various genera, including *Salmonella* (Freestone et al. 1999, 2007a, b; Williams et al. 2006), *Shigella* (O'Donnell et al. 2006), *Yersinia* (Freestone et al. 1999, 2007a, b; Lyte and Ernst 1992), *Vibrio* (Nakano et al. 2007b), *Campylobacter* (Cogan et al. 2007) as well as pathotypes of *E. coli* such as EHEC and ETEC (Lyte et al. 1997a). Norepinephrine can also resuscitate *E. coli* and *Salmonella* from a viable but nonculturable state (Reissbrodt et al. 2002), indicating that it may aid the outgrowth of physiologically-stressed bacteria that may enter mammalian hosts from the environment.

Systemic translocation of gut-derived bacteria and sepsis are well-known complications of surgery (reviewed in Nieuwenhuijzen and Goris 1999), and it is believed that outgrowth of enteric bacteria in response to the release of stress-related catecholamines may be significant in this process. Increased translocation of gut-derived bacteria to inguinal and mesenteric lymph nodes and the liver has also been observed during social conflict and restraint in C57BL/6 mice (Bailey et al. 2006). In addition, mice subject to partial hepatectomy are more susceptible to gut-derived sepsis caused by the opportunistic pathogen *Pseudomonas aeruginosa* (Laughlin et al. 2000) and exhibit elevated levels of NE in faeces (Alverdy et al. 2000). This was correlated with increased expression of the *Ps. aeruginosa* PA-I lectin in vivo, which is vital for pathogenesis (Alverdy et al. 2000; Laughlin et al. 2000). Expression of PA-I lectin is sensitive to NE in vitro (Alverdy et al. 2000) suggesting a molecular link between NE release under stress and the activity

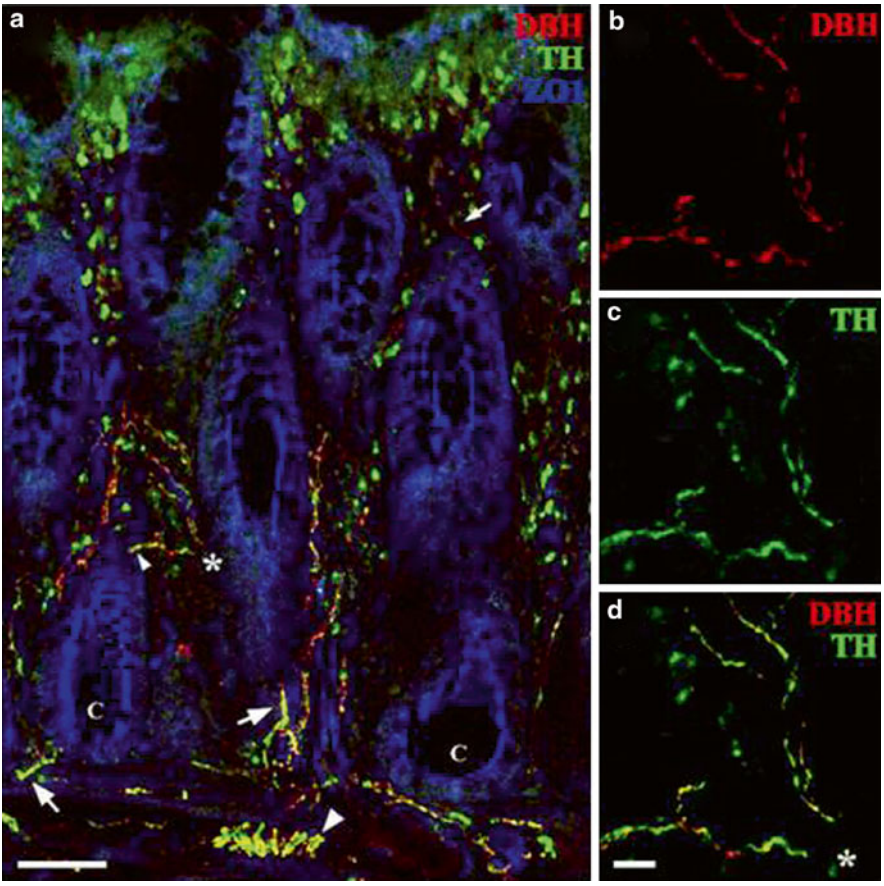

Fig. 6.1 Noradrenergic innervation in an oblique longitudinal section of porcine distal colon. (**a**) Colonic mucosa was triple-labelled for dopamine β-hydroxylase (DBH; *red*), tyrosine hydroxylase (TH; *green*), and the tight-junction protein zonula occludens-1 (ZO1; *blue*) to visualise epithelial cells. Fibres reactive to antibody against DBH and TH were present in the colonic villi and often terminated in close proximity to epithelial cells (*small arrowhead*). Only very fine fibres could be distinguished in the most apical portion of the villi (*small arrow*). Colocalisation of DBH and TH (*yellow*) was evident in submucosal ganglia (*large arrowhead*) and nerve bundles (*large arrow*) near crypts (**c**) at the base of the villi. (**b–d**) Imaging at high magnification demonstrated reactivity to antibodies against DBH (**b**) and TH (**c**) colocalised in varicose nerve fibres (**d**). *Asterisks* in **a** and **d** indicate the region of **a** (scale bar 100 μm) presented in **b–d** (scale bar 10 μm). The image is a projection of six optical sections collected at 1 μm intervals. Reprinted from Green et al. (2004)

of bacteria in the intestinal lumen. Soluble factors in filtrates of faeces from mice subject to partial hepatectomy are able to activate PA-I lectin expression in vitro (Wu et al. 2003), though the role of NE in such stimulation has yet to be formally proven. The endogenous opioid dynorphin, which is released from the intestinal mucosa in mice subject to ischemia/reperfusion injury, also enhanced *Ps. aeruginosa* virulence in this model and virulence gene expression in vitro and in vivo (Zaborina et al. 2007). A detailed review of the ability of *Ps. aeruginosa* to respond

to cues from the host may be found in Chap. 9. The following sections will focus on the impact and mode of action of stress-related catecholamines on the interaction of *E. coli* and *Salmonella* with intestinal mucosa.

6.2 Impact of Catecholamines on the Interaction of *E. coli* with Intestinal Mucosa

As indicated earlier, increased faecal excretion of *E. coli* has been noted in pigs subject to social stress (Jones et al. 2001) or physical handling (Dowd et al. 2007). Evidence that such events may be correlated with the release of stress-related catecholamines is afforded by the observation that the selective neurotoxin 6-hydroxydopamine rapidly and dramatically increases the number of *E. coli* in the cecum of mice (Lyte and Bailey 1997). 6-hydroxydopamine destroys noradrenergic nerve terminals without transit across the blood–brain barrier and elicits an immediate systemic release of NE. Within 14 days, during which time noradrenergic nerves regenerate, coliform counts returned to normal (Lyte and Bailey 1997). The effect could be inhibited by prior administration of desipramine hydrochloride, which specifically inhibits catecholamine uptake into noradrenergic nerve terminals, implying that damage to such neurons is required for bacterial outgrowth (Lyte and Bailey 1997). In murine models, stress induced by partial hepatectomy or short-term starvation also caused a profound increase in the number of *E. coli* adhering to the cecal mucosa compared to control mice (Hendrickson et al. 1999).

In addition to evidence that release of endogenous catecholamines may alter the activities of luminal *E. coli*, direct instillation of NE into the gut lumen has been reported to enhance adherence of *E. coli* O157:H7 to the mucosal surface in a bovine ligated ileal loop model (Vlisidou et al. 2004). *E. coli* O157:H7 can rarely be found in association with the mucosa 12 h post-inoculation of bovine ileal loops (Fig. 6.2a), however, if combined with 5 mM NE immediately prior to inoculation, dense microcolonies of adherent bacteria could be observed (Fig. 6.2b). Adherent bacteria were found to have formed “attaching and effacing” (AE) lesions that are known to be vital for bacterial persistence in the bovine intestines (Fig. 6.2c; Dziva et al. 2004; van Diemen et al. 2005). NE also significantly increased fluid accumulation and the recruitment of ^{111}In oxinate-labelled neutrophils in response to *E. coli* O157:H7 compared to control loops filled with bacteria and diluent, albeit only when NE used at 5 mM and not 50 μM (Vlisidou et al. 2004). NE alone did not induce enteritis in this model, indicating that the effect was not the result of outgrowth of resident enteric bacteria or damage to intestinal mucosa.

The relevance of observations in the ligated loop model, where millimolar concentrations of NE were used, to the activities of *E. coli* in a stressed host is unclear. Short-term starvation prior to surgery and manipulation of the intestines to construct ligated segments does not stimulate extensive adherence of *E. coli* O157:H7 per se (Fig. 6.2a), and one may therefore question the relevance of the observations. However, the concentration of NE in the intestinal tract of normal and surgically

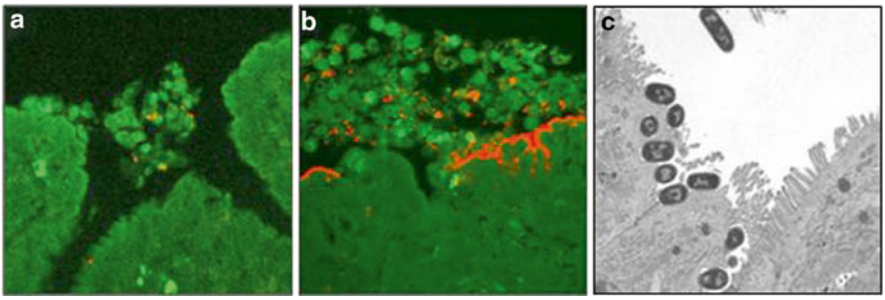

Fig. 6.2 Confocal laser scanning micrographs of bovine mid-ileal mucosa from ligated loops inoculated with *E. coli* O157:H7 strain 85–170 nal^R in the presence of diluent (**a**), or 5 mM NE (**b**). F-actin was stained with fluorescein isothiocyanate-conjugated phalloidin (*green*), and bacteria detected with rabbit anti-O157 typing serum and anti-rabbit Ig-$Alexa^{568}$ (*red*). Dense microcolonies of intimately attached bacteria were seen only in the presence of NE. Magnification ×630. Increased recruitment of neutrophils in the presence of NE was confirmed by analysis of γ-emissions associated with ^{111}In-labelled neutrophils in the same tissues as used for microscopy (Vlisidou et al. 2004). (**c**) Shows a transmission electron micrograph of AE lesions induced by *E. coli* O157:H7 in the presence of 5 mM NE. Scale bar = 5 μm. Reprinted from Vlisidou et al. (2004)

manipulated calves is unknown. Quantification of tissue-associated and free NE in the intestines is difficult for several reasons (Grassi and Esler 1999; Hjemdahl 1993); (1) microdialysis probes used to sample NE would not be suitable for use in the gut owing to blockage of the dialysis membrane and the fact that NE levels in the dialysate may not reach equilibrium with the surroundings over time, (2) high-pressure liquid chromatography is required to quantify NE in gut contents and recovery through purification steps cannot be estimated, (3) breakdown products of NE can be detected in the intestines, and it is not feasible to calculate the loss of NE through the activity of host and/or bacterial enzymes or other processes. Despite these limitations, the model offers the advantage that at least ten strains or treatments can be evaluated in triplicate in the same animal relative to internal positive and negative controls, thereby minimising the impact of inter-animal variation.

Analysis of muscle-stripped intestinal explants clamped between half-tubes in Ussing chambers provides a further tractable model to probe the role of bacterial and host factors in colonization of the mucosal surface (reviewed in Chap. 5). In this system, NE promotes adherence of *E. coli* O157:H7 to murine cecal mucosa (Chen et al. 2003) and porcine colonic mucosa (Green et al. 2004). NE also promoted uptake of *E. coli* O157:H7, but not a rodent commensal *E. coli* strain, into porcine jejunal Peyer's Patch tissue. EHEC are not widely considered to be invasive pathogens, and uptake by such tissue may reflect the antigen-sampling activity of M cells in follicle-associated epithelium (FAE). It is therefore noteworthy that FAE derived from rats subjected to chronic psychological (water avoidance) stress was found to take up higher levels of fixed fluorophore-conjugated *E. coli* K-12 as

compared to epithelium from control rats (Velin et al. 2004). As inactivated bacteria were used and explants were not treated with exogenous neurochemicals, the data imply that stimulation of host tissues under stress may modulate subsequent interactions with enteric bacteria. Increased bacterial uptake was not observed in villus epithelium from stressed rats, indicating that the effect may be specific to FAE.

It is important to note that NE-stimulated adherence of *E. coli* O157:H7 to the luminal aspect of explants clamped in Ussing chambers occurred following the application of NE to the contraluminal aspect. Though it is not possible to preclude the possibility that some NE diffused to the luminal side, the amount of diffused NE and time of exposure is considered unlikely to have been adequate to promote a substantial increase in the number of bacteria available for adherence. Measurements of short circuit current and tissue electrical conductance indicated that the mucosa was viable and intact. In addition, pre-treatment of murine cecal explants with either the non-selective α-adrenergic receptor antagonist phentolamine or the β-adrenergic receptor antagonist propranolol prevented the action of NE, indicating that NE-promoted bacterial adherence at least partially reflects alterations in the host tissue (Chen et al. 2003). A similar effect of phentolamine on adherence and entry of *E. coli* O157:H7 into porcine jejunal Peyer's patch mucosa has been reported (Green et al. 2003), however in this system, propanolol did not inhibit the effect of NE (Green et al. 2004; Chen et al. 2006). Evidence exists that the ability of NE to stimulate adherence of EHEC to porcine intestinal explants may be more sensitive to the α_2-adrenergic receptor antagonist yohimbine than the α_1-adrenergic antagonist prazosin (Green et al. 2004; Chen et al. 2006). The importance of release of NE from endogenous stores has also been suggested by the use of sympathomimetic drugs. For example, the NE reuptake inhibitor cocaine and α_2-adrenergic receptor agonist UK-14,304 both stimulate adherence of *E. coli* O157:H7 to porcine colon explants (Chen et al. 2006), as does a combination of the NE reuptake blocker desipramine and pargyline, an inhibitor of monoamine oxidase that influences catecholamine metabolism (Green et al. 2004).

Though most studies with explants have focussed on NE, it has also been shown that DA increases adherence of *E. coli* O157:H7 to murine cecal mucosa when applied to the contraluminal aspect in a manner sensitive to the DA antagonist haloperidol (Chen et al. 2003). The stress-related peptide adrenocorticotropic hormone (ACTH) also augments adherence of *E. coli* O157:H7, but not a pig-adapted non-pathogenic *E. coli*, to porcine colonic mucosa (Schreiber and Brown 2005). Tyramine, which is structurally related to dopamine and abundant in certain dairy products owing to the tyrosine decarboxylase activity of *Enterococcus faecalis* starter cultures, can promote adherence of *E. coli* O157:H7 to murine cecal explants (Lyte 2004). In addition, extracts of banana that are rich in NE and serotonin (Waalkes et al. 1958) augment growth of Gram-negative bacteria (Lyte 1997), as do a number of non-catecholamine dietary catechols (Freestone et al. 2007c; reviewed in Chap. 4). Further studies are required to determine if neurochemicals consumed in the diet are able to modulate the outcome of enteric bacterial infections.

6.2.1 Possible Mechanisms of Action of Norepinephrine During E. coli Infection

Though evidence exists that catecholamines partly exert their effects by acting on host tissues (above), it is clear that during culture in vitro they promote the growth of Gram-negative bacteria of several genera and expression of their virulence factors. *E. coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica* vary markedly in their ability to grow in response to catecholamines (Freestone et al. 2007b). The response of *Y. enterocolitica* was limited to NE and DA, and these proved to be more potent inducers of growth than epinephrine in *E. coli* and *Salmonella*. The impact of catecholamines on bacterial growth is strictly dependent on inoculum density and media composition, being most prominent in a minimal salts medium supplemented with 30% (v/v) adult bovine serum (serum-SAPI) from inocula of 10^2–10^3 colony-forming units (CFU; reviewed in Chap. 3). From low inocula such media are bacteriostatic in the absence of catecholamines and are proposed to mimic the nutrient poor and iron-limited conditions encountered in vivo. The effect of catecholamines on growth is ameliorated during culture in rich media or at high inoculum densities. Growth stimulation is unlikely to be due to provision of a metabolite, as supplementation with the NE derivative normetanephrine, which contains one more methyl group than NE, failed to produce the effect (Lyte et al. 1996a, 1997a, b). Remarkably, α- (but not β-) adrenergic receptor antagonists are able to block NE- and epinephrine-induced growth and dopaminergic receptor antagonists inhibit the growth response to DA in the absence of effects on cell viability (Freestone et al. 2007a). These data imply that Gram-negative bacterial pathogens may possess elements that specifically interact with catecholamines and/or that antagonists can interfere with the ability of catecholamines to liberate factors required for bacterial growth.

Two major hypotheses have been put forward to explain the ability of NE to promote growth and virulence gene expression, and these are not mutually exclusive. The first posits that NE and related catecholamines facilitate the supply of iron to Gram-negative bacteria under iron-limiting conditions. As many virulence genes in Gram-negative bacteria are growth-phase regulated, this may explain downstream effects. The second hypothesis posits that virulence gene expression may result from microbial detection of catecholamines via specific receptors that initiate a signal transduction cascade on receipt of the signal leading to altered gene expression (Fig. 6.3). These hypotheses are considered in more detail subsequently.

The ability to liberate ferric iron from host storage proteins such as lactoferrin (Lf) and transferrin (Tf) is a key requirement in bacterial pathogenesis. Many Gram-negative bacteria secrete low molecular weight catecholate or hydroxamate siderophores to acquire iron, which are then imported via specific receptors. Significantly, NE, epinephrine and DA appear to facilitate iron supply from Lf and Tf, likely via the ability of the catechol moiety to complex ferric ion thereby lowering its affinity with Lf and Tf and releasing iron for siderophores to capture (Freestone et al. 2000, 2002, 2003). Recent data indicate that the formation of NE

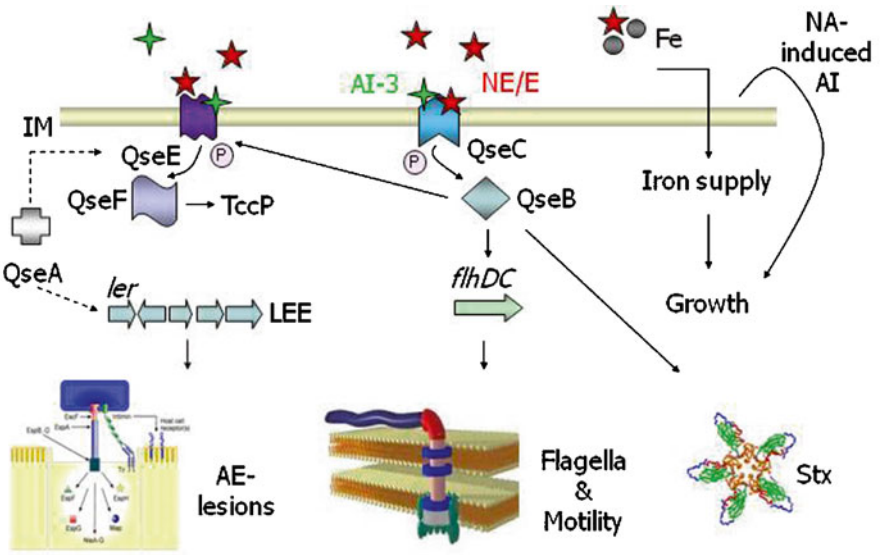

Fig. 6.3 Catecholamine sensing and signal transduction in *E. coli* O157:H7. QseC is an adrenergic sensor kinase that autophosphorylates on detection of epinephrine/NE/AI-3 and transfers the phosphate moiety to its cognate response regulator QseB, thereby activating transcription of the flagellar regulon (Clarke et al. 2006). The signalling cascade downstream of QseC in *E. coli* O157:H7 is known in some detail and is discussed elsewhere (Hughes et al. 2009). Transcription of genes encoding a second two-component system (QseEF) is sensitive to epinephrine and QseC (Reading et al. 2007). QseEF influences AE-lesion formation via activation of genes encoding a Type III secretion system and the Tir-cytoskeleton coupling protein (Reading et al. 2007), and recent evidence indicates that it directly senses catecholamines (Reading et al. 2009). Catecholamines also mediate iron supply by an ill-defined mechanism and promote bacterial replication in serum-rich iron-limited media from low inoculum densities by a mechanism that may involve induction of a heat-stable autoinducer (AI). NE stimulates production of Shiga toxins in *E. coli* O157:H7 however the signal transduction cascade leading to this event is unclear at the time of writing. Adapted from Reading et al. (2007)

complexes with Lf and Tf leads to reduction of Fe(III) to Fe(II), for which Lf/Tf have a lower affinity (Sandrini et al. 2010). Siderophore synthesis and transport are required for NE-stimulated growth of *E. coli*, as strains with mutations affecting enterobactin synthesis (*entA*) or ferric-enterobactin transport (*fepA* or *tonB*) do not respond to NE in iron-limited serum-rich medium from a low inoculum (Burton et al. 2002; Freestone et al. 2003). This implies that NE does not act as a siderophore per se, although tritiated NE is taken into *E. coli* cells (Freestone et al. 2000; Kinney et al. 2000) and the role played by NE-ferric ion complexes in growth induction and the mechanism of NE import remain unclear. NE has also been found to induce the production of the ferric-enterobactin receptor (FepA), indicating that it may facilitate iron acquisition by multiple mechanisms (Burton et al. 2002).

Iron supply may not be the only mechanism by which catecholamines induce bacterial growth. Supernatants of *E. coli* cultures collected after induction of growth by NE in iron-limited serum-rich medium from low inocula contain a heat-stable autoinducer of growth that stimulates replication of naïve bacteria to a comparable extent as catecholamines (Lyte et al. 1996b; Freestone et al. 1999). The autoinducer is produced rapidly after exposure to NE and is able to activate growth of Gram-negative bacteria of several genera (Lyte et al. 1996b; Freestone et al. 1999). It has also been implicated in the ability of NE to resuscitate viable but non-culturable *E. coli* and *Salmonella* and appears to act in manner independent of iron-supply from Lf and Tf (Reissbrodt et al. 2002).

The ability of catecholamines to promote interactions between pathogenic *E. coli* and intestinal mucosa may reflect not only bacterial outgrowth, but also the induction of virulence factors. In ETEC O9:K30:H-, NE promotes expression of the K99 pilus adhesin (Lyte et al. 1997a, b). K99 pili are important in the pathogenesis of ETEC-induced diarrhoea in pigs, and such regulation may partially explain the effect of stress on the outcome of ETEC infection in pigs (Jones et al. 2001). Increased expression of Type I pili has also been described in commensal *E. coli* following stress induced by partial hepatectomy or short-term starvation in mice (Hendrickson et al. 1999). Such fimbriae are vital in the pathogenesis of ascending urinary tract infections by *E. coli* and were proposed to mediate important interactions with intestinal epithelia following catabolic stress (Hendrickson et al. 1999). However, this does not offer an explanation for the increased adherence of *E. coli* O157:H7 to bovine intestinal mucosa in the presence of NE (Vlisidou et al. 2004; Fig. 6.2b), as *E. coli* O157:H7 fail to elaborate functional Type I fimbriae owing to mutations in the *fimA* promoter (Roe et al. 2001) and FimH adhesin (Bouckaert et al. 2006). Indeed, expression of Type I pili is negatively selected during colonization of the bovine intestines by EHEC O26:H- (van Diemen et al. 2005).

Norepinephrine has also been reported to stimulate the production of Shiga toxins by *E. coli* O157:H7 during growth from a low inoculum in serum-containing medium (Lyte et al. 1996a, 1997a). Toxin production could also be stimulated by the catecholamine-induced autoinducer of growth, indicating that NE may partially act via this secreted intermediary (Voigt et al. 2006). Enhanced production of Shiga toxins is significant in the context of EHEC pathogenesis in humans, as the toxins may cause acute renal and neurological sequelae via damage to microvascular endothelial cells. Shiga toxins have also been reported to influence persistence of *E. coli* O157:H7 in the intestines of mice (Robinson et al. 2006) and rabbits (Ritchie et al. 2003), and to deplete a subset of intraepithelial lymphocytes in the bovine intestines (Menge et al. 2004). However, it is unlikely that this would explain the ability of NE to augment adherence and *E. coli* O157:H7-induced enteritis in bovine ligated ileal loops as a non-toxigenic strain was used and Shiga toxin 1 does not play a significant role in EHEC-induced enteritis in this model (Stevens et al. 2002).

A more plausible explanation for the ability of NE to stimulate adherence of *E. coli* O157:H7 to mucosa surfaces was afforded by the finding that it increases the production of several factors required for the formation of AE lesions

(Sperandio et al. 2003; reviewed in Chap. 12). AE lesions are characterised by intimate bacterial attachment to enterocytes and localised destruction of microvilli (Fig. 6.2c), and their formation relies on a Type III protein secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE). This apparatus injects a set of bacterial proteins into enterocytes, one of which (the translocated intimin receptor, Tir) becomes localised in the apical leaflet of the host cell plasma membrane where it serves as a receptor for the bacterial outer membrane protein intimin. Both intimin, Tir, and the T3SS play pivotal roles in colonization of the bovine intestines by *E. coli* O157:H7 (Dziva et al. 2004; van Diemen et al. 2005; Vlisidou et al. 2006). A mutant lacking both intimin and Tir did not adhere to the surface of bovine ileal loops in response to NE 12 h after loop inoculation (Vlisidou et al. 2004). However, studies in Ussing chambers have indicated that intimin and the T3SS component EspA are not required for NE-stimulated early non-intimate adherence to porcine intestinal explants (Chen et al. 2006). Production of LEE-encoded proteins is regulated by the growth phase, however induction of the expression and secretion of Type III secreted proteins, as well as induction of transcription of LEE operons, was reported to occur in the absence of effects on growth (Walters and Sperandio 2006). Epinephrine and NE also promote motility of *E. coli* O157:H7 (Sperandio et al. 2003), by a mechanism that appears to involve induction of the flagella regulon via the master regulators FlhDC (Clarke et al. 2006). In a bovine model, an *E. coli* O157:H7 *flhC* mutant was impaired in its ability to persist in the intestines, but a mutant lacking the flagellin subunit FliC was not (Dobbin et al. 2006), indicating that if NE acted via this circuit to promote adherence in the bovine intestines, it may have required *flhDC*-regulated genes other than flagella genes for the effect.

A key contribution to our understanding of host-microbe communication was the identification of an adrenergic receptor in *E. coli* O157:H7 (Clarke et al. 2006; reviewed in Hughes and Sperandio 2008). This arose from the finding that the *qseBC* genes, which regulate flagella-mediated motility (Sperandio et al. 2002), are required for the ability of epinephrine to stimulate motility (Sperandio et al. 2003). QseBC exhibit homology to two-component systems, which typically comprise a sensor kinase (SK) that autophosphorylates at a conserved histidine on receipt of a specific signal and a response regulator (RR) to which the phosphate moiety is transferred from the cognate SK. Phosphorylation of the RR alters its activity, leading to altered expression of genes under its direct or indirect control. It was subsequently proven that QseC binds tritiated NE and that it autophosphorylates in response to epinephrine and NE when reconstituted in lipid micelles (Clarke et al. 2006). Transfer of the phosphate moiety to QseB after epinephrine stimulation of QseC could be detected, and this is believed to modulate transcription of genes under the control of QseB, which include *flhDC* (Clarke et al. 2006). The signalling cascade downstream of QseC is now known is some detail (Hughes et al. 2009). Catecholamine binding to QseC and autophosphorylation could be blocked by the α-adrenergic receptor antagonist phentolamine, but not by the β-adrenergic antagonist propanolol (Clarke et al. 2006), implying that QseC may structurally mimic eukaryotic adrenergic receptors. QseC also senses a bacterial autoinducer (AI-3)

that provides a measure of population density (Sperandio et al. 2003). The interplay between such 'quorum sensing' systems and detection of host-derived catecholamines is reviewed in detail in Chap. 12.

The precise role of the QseBC system in activation of LEE genes remains to be determined. Interestingly, NE-stimulated adherence, production of LEE-encoded proteins and AE-lesion formation can be inhibited by the β-adrenergic receptor antagonist propanolol (Chen et al. 2003; Sperandio et al. 2003), even though this treatment does not impair QseC autophosphorylation in the presence of NE (Clarke et al. 2006). This finding, taken together with the fact that some enteric pathogens respond to NE but lack a QseC homologue (e.g. *Campylobacter jejuni* and *Yersinia enterocolitica*), may reflect the presence of other bacterial catecholamine receptors or alternative modes of action. In the case of *E. coli* O157:H7, it has been reported that a second two-component sensory system (QseEF), that is regulated by QseBC and required for AE-lesion formation (Reading et al. 2007), acts as a secondary receptor for epinephrine (Reading et al. 2009). It has been established that QseC is required for the induction of enteritis by rabbit enteropathogenic *E. coli* in infant rabbits (Clarke et al. 2006). However, mutations in two-component systems often have pleiotropic effects, and it remains to be shown that QseC is required for NE-induced phenotypes of *E. coli* O157:H7 in vivo. A specific inhibitor of QseC signalling (LED209) has recently been reported to interfere with actin nucleation by *E. coli* O157:H7 and the transcription of *flhDC* and *stx2A*; however, studies of its ability to control infection in infant rabbits were complicated by rapid adsorption of the inhibitor from the gut (Rasko et al. 2008).

In an attempt to define the global transcriptional response of enteric pathogens to catecholamines, microarray studies have been undertaken by several laboratories. Bansal et al. defined the transcriptome of *E. coli* O157:H7 during biofilm formation on glass wool during 7 h culture in Luria Bertani (LB)-glucose medium supplemented with 50 μM epinephrine or 50 μM NE. A total of 938 and 970 genes were differentially transcribed in response to epinephrine and NE, respectively, with 411 genes exhibiting the same pattern of transcription in response to both catecholamines (Bansal et al. 2007). The fact that epinephrine and NE exert different effects at the level of transcription is consistent with the observation that they do not stimulate the growth of *E. coli* O157:H7 to the same extent (Freestone et al. 2007a, b). Several genes involved in iron acquisition were upregulated in response to epinephrine and NE, however Shiga toxin and LEE genes were not found to be activated under the conditions used (Bansal et al. 2007), in contrast with observations at the protein level. Following growth in serum-SAPI medium in the presence of 50 mM NE from an inoculum of c. 10^6 CFU, Dowd detected differential regulation of 101 genes, including induction of genes involved in iron acquisition and the genes for intimin, T3SS components and Shiga toxins (Dowd 2007). Such changes mirror those seen at the protein level (Lyte et al. 1996a, 1997a; Voigt et al. 2006; Sperandio et al. 2003); however, other genes in the same operon as *eae* and *espAB* were not observed to be differentially regulated. The *fepA* gene was found to be repressed, in contrast with other studies (Burton et al. 2002), whereas induction of *feoB* and *fhuD* was also reported by Bansal et al. Culture of

an *E. coli* O157:H7 *luxS* mutant (unable to synthesize autoinducer) in Dulbecco's modified Eagle's medium with 50 μM epinephrine has also been reported to induce transcription of LEE, *stx2* and flagella genes (Kendall et al. 2007). A key consideration with studies of this nature is the effect of exogenous catecholamines on bacterial growth. If control and treated cultures are collected at different points in the growth cycle, it is difficult to interpret whether altered virulence gene expression is a direct consequence of catecholamine-mediated regulation (e.g. via QseBC and QseEF) or associated with the change of growth phase. A further consideration is that at high concentrations catecholamines may sequester iron leading to indirect activation of iron acquisition genes. Such aspects are reviewed in detail elsewhere (Freestone et al. 2008).

6.3 Impact of Catecholamines on the Interaction of *Salmonella* with Intestinal Mucosa

In addition to the finding that pigs subject to social stress or transport exhibit increased faecal excretion of *S. enterica* serovar Typhimurium (Isaacson et al. 1999; Callaway et al. 2006), several studies have addressed the impact of pre-treating *Salmonella* or the host with catecholamines on the outcome of infection. Culture of *S*. Typhimurium in serum-SAPI medium containing 2 mM NE increased colonisation of selected tissues following oral inoculation of piglets compared to bacteria grown in Luria Bertani medium in the absence of NE (Toscano et al. 2007). However, it is likely that the physiological status of bacteria grown in serum-rich minimal salts medium is different to that of bacteria cultured in rich media and, though comparable numbers of bacteria were administered, they may have differed in growth phase and gene expression. Indeed, studies in the author's laboratory failed to reproduce the effect of precultivation in the presence of NE when *S*. Typhimurium strain 4/74 was grown in LB ± 5 mM NE prior to inoculation of 6-week-old Large White pigs (Stevens MP, unpublished observations).

In relation to the role of stress-related catecholamines during systemic salmonellosis, it has been reported that NE promotes *Salmonella* encephalopathy in calves challenged orally with clinical isolates of serovars Enteritidis, Montevideo and Saintpaul associated with neurological disease. Calves given 45 μg/kg NE daily via the intramuscular route developed neurological signs following inoculation with these isolates and had evidence of bacterial replication in the brain, whereas control animals given a placebo were only positive for bacteria by faecal culture (McCuddin et al. 2008). In the same study however, daily intramuscular administration of NE did not enhance faecal excretion of *S*. Typhimurium DT104 and even decreased excretion of *S*. Dublin (McCuddin et al. 2008), albeit that the relative ability of NE deposited in muscle to act on bacteria at enteric and systemic sites in calves at this dose is ill-defined. Intra-gastric administration of NE to mice the day before inoculation with *S*. Typhimurium increased cecal colonisation and translocation to the liver in a dose-dependent manner (Williams et al. 2006). Similarly, chicks given

twice daily NE by crop instillation exhibited elevated levels of *S. enterica* serovar Enteritidis in the ceca and liver compared to controls (Methner et al. 2008).

Research in the author's laboratory has indicated that *S.* Typhimurium-induced fluid accumulation and ^{111}In-labelled neutrophil recruitment in bovine ligated ileal loops is increased by mixing the bacteria with NE immediately prior to inoculation (Pullinger et al. 2010). However, as with *E. coli* O157:H7, relatively high concentrations of NE (5 mM) were required to produce a statistically robust phenotype in this model. Using explants of porcine jejunal Peyer's patch mucosa clamped in Ussing chambers, addition of NE to the contraluminal aspect increased uptake of *S.* Choleraesuis in a phentolamine-sensitive manner (Green et al. 2003). Whilst this implies that NE may facilitate *S.* Choleraesuis invasion by a mechanism dependent on host adrenergic receptors, NE did not significantly enhance internalisation of *S.* Typhimurium DT104 by porcine jejunal Peyer's patch explants or ileal non-follicular mucosa in Ussing chambers (Schreiber et al. 2007). Indeed treatment of such explants with sympathomimetic drugs even decreased recoveries of *S.* Typhimurium DT104 (Brown and Price 2008). *S.* Typhimurium invades porcine intestinal mucosa more efficiently than *S.* Choleraesuis in Ussing chambers (Brown and Price 2008) and ileal loops (Paulin et al. 2007), and the effect of exogenous catecholamine on invasion may therefore be less pronounced if a finite level of uptake is possible under the assay conditions. Further studies are required to determine if there are strain- or serovar-specific mechanisms by which catecholamines may modulate the outcome of *S. enterica* infections.

Studies on the importance of release of catecholamines from endogenous stores on the virulence of *Salmonella* are lacking. A glucose analogue, 2-deoxy-D-glucose (2DG), previously shown to induce many of the hallmark parameters of physiological stress in pigs (Stabel 1999), failed to reactivate *S.* Choleraesuis at enteric or systemic sites when given to carrier animals (Stabel and Fedorka-Cray 2004), however catecholamine levels in the intestines or circulation of treated and control animals were not measured. Studies in the author's laboratory have indicated that administration of 40 mg/kg 6-hydroxydopamine intravenously to pigs 8 or 16 days after oral inoculation with *S.* Typhimurium caused a transient but statistically significant increase in the number of excreted *Salmonella* relative to infected pigs given diluent (Stevens MP, unpublished observations). This is consistent with the outgrowth of commensal coliforms described in 6-hydroxydopamine treated mice (Lyte and Bailey 1997).

6.3.1 Possible Mechanisms of Action of Norepinephrine During Salmonella Infection

As with *E. coli*, catecholamines have been proposed to supply iron to *Salmonella* and to act via an adrenergic receptor(s). In relation to iron, NE-induced growth of *S.* Typhimurium from low inocula in serum-SAPI medium requires the synthesis of enterobactin (Methner et al. 2008). However, mutants lacking the catecholate

receptors IroN or FepA that mediate uptake of enterobactin, salmochelin S4 and 2,3-dihydroxybenzoylserine (DHBS) still grow in serum-SAPI in response to NE (Williams et al. 2006), as do those defective in enterobactin secretion (EntS) or conversion of enterobactin to salmochelin S4 (IroB; Methner et al. 2008). This implies that neither secretion nor uptake of enterobactin or its derivative salmochelin S4 is required for NE-mediated iron supply and suggests the possibility that enterobactin precursors or degradation products may be required for the effect. A mutant lacking Fes, which hydrolyzes enterobactin to DHBS failed to grow in serum-SAPI in response to NE (Methner et al. 2008) as did a triple mutant lacking IroN, FepA and Cir, all of which act as receptors for DHBS (Williams et al. 2006). The reduced ability of a *S*. Typhimurium *fes* mutant and an *iroN fepA cir* triple mutant to grow in response to NE has been independently reported (Bearson et al. 2008). A mutant lacking IroD, which hydrolyses salmochelin S4 to the linear glycosylated monomeric form SX, also blocked the growth response to NE (Methner et al. 2008). These data support the notion that degradation products of enterobactin and salmochelin S4 may be important for NE-mediated iron supply to *Salmonella*. The relevance of such events in vivo requires further study as whilst inoculation of mice pre-treated with NE did not result in elevated growth of a triple *iroN fepA cir* mutant (Williams et al. 2006), a mutant lacking TonB, which is required for uptake via these receptors, behaved in a manner comparable to the parent strain (Methner et al. 2008). Additionally, an *iroN fepA cir* triple mutant has been reported to show no defect in colonisation of the porcine intestines (Bearson et al. 2008), in contrast to observations in mice (Williams et al. 2006).

S. Typhimurium encodes orthologues of the *E. coli* O157:H7 QseC adrenergic sensor kinase (also known as PreB, YgiY or STM3178 in *S*. Typhimurium), and QseB response regulator (PreA, YgiX, STM3177). Although the ability of these orthologues to sense and respond to epinephrine and NE has yet to be proven, evidence exists that they encode a functional two-component system (TCS) that upregulates transcription of the *pmrCAB* genes encoding a further TCS that in turn regulates virulence gene expression (Merighi et al. 2006). The *E. coli* O157:H7 *qseBC* genes were able to functionally replace *preAB* as regulators of *pmrCAB* transcription in *S*. Typhimurium (Merighi et al. 2006), however reciprocal studies in *E. coli* have yet to be reported. Recent studies have indicated that the QseC orthologue in *S*. Typhimurium is required for full systemic virulence in mice (Merighi et al. 2009; Moreira et al. 2010) and influences NE-induced motility, invasion of IPEC-J2 porcine jejunal cells and intestinal colonisation of swine (Bearson and Bearson 2008). Further, the inhibitor of *E. coli* O157:H7 QseC signalling LED209 aids control of systemic salmonellosis in a murine model (Rasko et al. 2008). However, in calves only modest attenuation of a *S*. Typhimurium *qseC* mutant was observed (Pullinger et al. 2010). Two-component sensory systems are known to integrate multiple signals (indeed QseBC detects epinephrine, NE and AI-3), and evidence that QseC is required for full virulence does not provide evidence that catecholamine-sensing is required for full virulence. Indeed, recent studies have indicated that norepinephrine augments *S*. Typhimurium-induced enteritis in a bovine ligated ileal loop model of infection

independently of QseC, QseE or both putative sensor kinases (Pullinger et al. 2010). The authors of this report also examined net replication of the bacteria using a plasmid partitioning system previously used to study in vivo growth of *S. enterica* serovars (Paulin et al. 2007). NE treatment enhanced net replication of *S*. Typhimurium in the same intestinal segments as it stimulated enteritis, consistent with NE-induced effects on growth in vitro (Pullinger et al. 2010).

NE has been reported to activate expression of the *S*. Typhimurium Type III secretion-related genes in a manner sensitive to LED209 and mutation of *qseC* (Rasko et al. 2008; Moreira et al. 2010). Type III secretion systems-1 and -2 are vital for persistence in the mammalian intestines and induction of enteritis (reviewed in Stevens et al. 2009). However, recent microarray studies of the QseBC regulon (Merighi et al. 2009) and the response of *S*. Typhimurium to epinephrine (Karavolos et al. 2008) do not support effects on Type III secretion loci. Stimulation of *S*. Typhimurium with NE also failed to induce the production or secretion of T3SS-1 or T3SS-2 proteins as detected by western blotting and use of translational fusions (Pullinger et al. 2010). T3SS-1 expression is sensitive to the availability of exogenous iron, being decreased in the presence of the iron chelator dipyridyl (Ellermeier and Slauch 2008). Thus the ability of NE to augment *Salmonella*-induced enteritis in ileal loops is unlikely to be due to T3SS-1 induction as a consequence of sequestering free iron by use of high concentrations of NE. It remains possible that differences in the role or mode of action of adrenergic sensor kinases may exist between *E. coli* and *Salmonella*, and functional redundancy may mask the effect of mutations studied to date. *S*. Typhimurium genes regulated by NE or epinephrine have recently been identified by a genome-wide transposon mutagenesis screen (Spencer et al. 2010), and further studies are required to understand the basis of NE-induced phenotypes in *Salmonella* and the basis of discrepancies between laboratories.

6.4 Modulation of the Activities of Other Enteric Pathogens by Catecholamines

It is becoming clear that other enteric bacterial pathogens possess the ability to respond to norepinephrine. NE promotes growth of *Campylobacter jejuni* in iron-limited media and bacterial uptake of ^{55}Fe (Cogan et al. 2007), as well as growth of the periodontal isolate *C. gracilis* (Roberts et al. 2002). *C. jejuni* lacks known siderophores but encodes homologues of siderophore receptors and it has been proposed that NE may bind iron and supply it to *Campylobacter* directly. In contrast to observations in *E. coli* (Freestone et al. 2000), no uptake of tritiated NE into *C. jejuni* cells could be detected (Cogan et al. 2007). Growth of *C. jejuni* in NE-supplemented medium markedly enhanced its ability to invade Caco-2 cell monolayers and disrupt tight junctions, as detected by a decrease in transepithelial electrical resistance and redistribution of occludin (Cogan et al. 2007). This may partially be explained by the ability of NE to promote motility of *C. jejuni* (Cogan

et al. 2007), which mediates invasion of epithelial cells in vitro and in vivo. Stimulation of *C. jejuni* growth by NE is insensitive to the adrenergic receptor antagonists phenoxybenzamine, propanolol and metoprolol tartrate (Cogan et al. 2007) and this, taken together with the fact that *C. jejuni* lacks known orthologues of the QseC and QseE adrenergic sensor kinases (Clarke et al. 2006), implies that NE may stimulate growth and gene expression in *Campylobacter* by distinct mechanisms. The impact of stress, whether induced during commercial practices or experimentally, on the carriage and virulence of *Campylobacter* requires further study. If correlated, analysis of bacterial gene expression in response to mediators of the host stress response and studies on the involvement of catecholate receptors in catecholamine-promoted phenotypes would be valuable.

In *Vibrio* species, NE, epinephrine and dopamine were found to promote growth of *V. parahaemolyticus* and *V. mimicus* in serum-SAPI medium, but not *V. cholerae* or *V. vulnificus* (Nakano et al. 2007b). *V. vulnificus* could be stimulated to grow in serum-SAPI with high concentrations of epinephrine, indicating that the specificity and magnitude of the response of catecholamines varies among *Vibrio* species (Nakano et al. 2007b), as described for *S. enterica*, *E. coli* and *Y. enterocolitica*. Stimulation of the growth of *V. parahaemolyticus* appears not to be due to provision of energy, as NE metabolites failed to elicit the effect (Nakano et al. 2007b). Although the effect of catecholamines on growth of the aquatic pathogen *V. splendidus* was not examined in this study, stress associated with physical shaking of oysters (*Crassostrea gigas*) challenged with *V. splendidus* increased both bacterial loads and mortality (Lacoste et al. 2001). Furthermore, physical stress was associated with elevated levels of circulating NE and injection of NE or adrenocorticotropic hormone, a key component of the oyster stress response, caused significantly higher mortality and increased accumulation of *V. splendidus* in challenged oysters (Lacoste et al. 2001). Although the molecular basis of this phenomenon is ill-defined, NE has been shown to increase the transcription of Type III secretion genes in *V. parahaemolyticus* and to augment cytotoxicity against Caco-2 cells and fluid accumulation in rat ligated ileal loops (Nakano et al. 2007a). NE-promoted fluid accumulation could be inhibited by phentolamine and the α_1-specific antagonist prazosin (Nakano et al. 2007a). As with *E. coli* and *Salmonella*, it remains unclear to what extent NE and adrenergic receptor antagonists exert their effect by acting on the bacteria or host.

6.5 Concluding Remarks

Enteric bacterial pathogens of many genera respond to catecholamine mediators of the host stress response. Whilst, the molecular mechanisms by which *S. enterica* and *E. coli* respond to catecholamines are rapidly being unravelled, the modes of iron supply and catecholamine sensing and signal transduction in other NE-responsive genera such as *Campylobacter*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Hafnia*, *Listeria*, *Shigella*, *Vibrio* and *Yersinia* are unknown. Further, the impact of stress-related

catecholamines on gene expression in such genera and its relationship to the outcome of infection in stressed hosts is ill-defined. Whilst, the involvement of selected catecholate receptors in NE-promoted virulence of *S*. Typhimurium in mice and chickens has been probed, it remains to be proven that adrenergic sensors act to promote bacterial virulence because they sense catecholamines, as opposed to detection of other cues or activation of unrelated regulons. Care also needs to be taken in the design of experimental animal studies in relation to the physiological status of catecholamine-treated and untreated bacteria and interpretation of phenotypes resulting from high exogenous catecholamine doses.

It is clear that stress-related catecholamines may act via a number of mechanisms, both in relation to bacteria, where they may promote iron-acquisition, autoinducer production and signal transduction, and in relation to the host. Further research will be needed to evaluate the effects of stress-related catecholamines on epithelial and immune cells in the gut, and the ability of adrenergic receptor antagonists and sympathomimetic drugs to modulate subsequent interactions between the mucosa and enteric bacteria. Molecules that interfere with the dialogue between pathogen and host may represent a novel class of anti-infective agents (Rasko et al. 2008). However, highly selective agents will likely be needed as the commensal microflora is also sensitive to stress-related catecholamines and plays a vital role in intestinal development and homeostasis. Analysis of microflora dynamics and the frequency of enteric infection in humans given adrenergic receptor antagonists for the treatment of hypertension, heart failure, migraine and other conditions may provide valuable data for the development of such agents. In addition, reporter systems that provide a measure of adrenergic stimulation of bacterial growth or virulence gene expression will be useful for screening chemical libraries. Although many genera respond to catecholamines, the specificity and magnitude of the response may vary and thus solutions may need to be tailored to the pathogen. Nevertheless, work in this area is given impetus by the decline in discovery of new antibiotics, rise in antibiotic resistance and the fact that antibiotic treatment of some enteric infections is contraindicated.

Whilst microbial detection of stress-related catecholamines enjoys a high profile at present, the full extent of microbial cross-talk with the host remains unclear. *Ps. aeruginosa* responds to the endogenous opioid dynorphin (Zaborina et al. 2007), and it is noteworthy that the *E. coli envY* gene reportedly encodes a functional high-affinity opioid receptor (Cabon et al. 1993). Endogenous opioids are among the first signals to be released by tissues under stress and the relevance of EnvY during enteric bacterial infection requires further study. In addition, evidence is emerging that bacterial pathogens may sense the activation of host innate immunity. For example the pro-inflammatory cytokine interferon-γ binds the *Ps. aeruginosa* outer membrane porin OprF and activates PA-I lectin expression (Wu et al. 2005), tumour necrosis factor-α increases invasion by *Shigella flexneri* (Luo et al. 1993) and interleukin-1 stimulates growth of pathogenic *E. coli* (Porat et al. 1991). Such findings suggest that the interplay between bacterial pathogens and their hosts may be far more complex than at first thought and have far reaching implications in human and veterinary medicine.

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Chapter 7
Molecular Profiling: Catecholamine Modulation of Gene Expression in *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium

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Abstract Investigations of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium have demonstrated that these bacterial pathogens can respond to the presence of catecholamines including norepinephrine and/or epinephrine in their environment by modulating gene expression and exhibiting various phenotypes. For example, one of the most intensively investigated phenotypes following exposure of *E. coli* and *S.* Typhimurium to norepinephrine is enhanced bacterial growth in a serum-based medium. Host-pathogen investigations have demonstrated that the mammalian host utilizes nutritional immunity to sequester iron and prevent extraintestinal growth by bacterial pathogens. However, *Salmonella* and certain *E. coli* strains have a genetic arsenal designed for subversion and subterfuge of the host. Norepinephrine enhances bacterial growth due, in part, to increased iron availability, and transcriptional profiling indicates differential expression of genes encoding iron acquisition and transport proteins. Bacterial motility of *E. coli* and *S.* Typhimurium is also enhanced in the presence of catecholamines and increased flagellar gene expression has been described. Furthermore, epinephrine and norepinephrine are chemoattractants for *E. coli* O157:H7. In *S.* Typhimurium, norepinephrine enhances horizontal gene transfer and increases expression of genes involved in plasmid transfer. Exposure of *E. coli* O157:H7 to norepinephrine increases expression of the genes encoding Shiga toxin and operons within the locus of enterocyte effacement (LEE). Alterations in the transcriptional response of enteric bacteria to catecholamine exposure in vivo are predicted to enhance bacterial colonization and pathogen virulence. This chapter will review the current literature on the transcriptional response of *E. coli* and *S.* Typhimurium to catecholamines.

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7.1 Introduction

The ability of bacteria to respond to the catecholamines epinephrine (Epi) and norepinephrine (NE) has stimulated intense interest in the field of microbial endocrinology for greater than a decade. In the family *Enterobacteriaceae*, *Escherichia coli* and *Salmonella enterica* have been investigated most frequently. Multiple phenotypes have been described for both *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) in response to Epi and NE. The utilization of microbial endocrinology by *Salmonella* and certain *E. coli* strains to acquire iron in vivo and subvert nutritional immunity imposed by the mammalian host is perhaps the phenotype that is best understood. However, additional phenotypes including Epi-/NE-enhanced motility, virulence gene regulation, and horizontal gene transfer are equally intriguing. This chapter will focus on the current knowledge of transcriptional profiling in *Escherichia coli* O157:H7 (O157:H7) and *S.* Typhimurium.

7.2 *Salmonella enterica*

7.2.1 Experimental Conditions

Multiple laboratories have investigated the response of *Salmonella enterica* serovar Typhimurium to the catecholamines epinephrine or norepinephrine. These investigations often utilized different strain backgrounds, media, and different concentrations of catecholamines. For example, the concentration of NE in different investigations ranges from 5 μM to 5 mM. Therefore, a direct comparison between individual experiments is difficult and may not be scientifically appropriate. Pullinger et al. (Pullinger et al. 2010a) utilized serum-SAPI medium with 5 mM NE and Dulbecco's modified Eagle's medium (DMEM) containing either 5 or 50 μM NE. Investigations by Bearson and colleagues utilized serum-SAPI medium containing 50 μM or 2 mM NE and DMEM motility medium containing 50 μM or 100 μM NE (Bearson and Bearson 2008; Bearson et al. 2008; Bearson et al. 2010). Karavolos et al. utilized LB medium containing 50 μM adrenaline (epinephrine, EPI) (Karavolos et al. 2008). Merighi et al. utilized DMEM motility medium containing 50 μM Epi (Merighi et al. 2009). The Sperandio laboratory has utilized LB medium containing 50 μM NE (Moreira et al. 2010; Rasko et al. 2008) and LB and N-minimal medium containing 50 μM Epi (Moreira and Sperandio 2012).

7.2.2 The QseBC Two-Component System

The regulatory system most frequently investigated in catecholamine-enhanced phenotypes is the QseBC two-component system with QseC as the sensor kinase and QseB serving as the response regulator. A point of contention in this subject area concerns the role of the QseBC two-component system in NE and Epi signaling; specifically at least four laboratories have investigated the response of *S.* Typhimurium motility to catecholamines without a clear consensus concerning whether QseC is involved. For example, Bearson et al. indicated that wild-type *S.* Typhimurium, as well as *qseC*, *qseB*, and *qseBC* mutants have a significant increase in motility on DMEM in response to 50 μM NE (Bearson et al. 2010). However, although motility of a *qseC* mutant significantly increases in response to NE exposure, the motility of a *qseC* mutant is significantly decreased compared to wild-type in the presence or absence of 50 μM NE. The bacterial motility of *qseB* and *qseBC* mutants are either similar or slightly greater than wild-type in both the presence or absence of 50 μM NE. Therefore, data from Bearson et al. indicates that the QseB response regulator is a negative repressor of bacterial motility with the motility repression phenotype of the QseBC two-component system dependent upon the presence of QseB in the absence of *qseC*, but NE-enhanced motility of *S.* Typhimurium did not require QseBC (Bearson et al. 2010). Moreira et al. demonstrated that *S.* Typhimurium motility was significantly enhanced on LB medium in the presence of 50 μM NE; the Authors indicate that NE enhanced the bacterial motility of a *qseC* mutant, but this difference was not statistically significant compared to the absence of NE (Moreira et al. 2010). Merighi et al. demonstrated that, although 50 μM Epi enhanced the motility of both wild-type *S.* Typhimurium and a *qseC* (*preB*) mutant on DMEM, this difference was not statistically significant (Merighi et al. 2009). However, this study did indicate that Epi-enhanced motility was statistically significant for both *qseB* (*preA*) and *qseBC* (*preAB*) mutants. Due to the Epi-enhanced motility of the *qseBC* mutant on DMEM, Merighi et al. indicated that the Epi response was not mediated by QseB/QseC. An investigation by Pullinger et al. indicated that in contrast to some of the above mentioned studies, neither wild-type *S.* Typhimurium nor a *qseC* mutant consistently responded to NE on DMEM motility medium (Pullinger et al. 2010a). Furthermore, the *qseC* mutant investigated by Pullinger et al. did not have decreased motility compared to wild-type *S.* Typhimurium, another contrast from other publications. One potential reason for discrepancies concerning the role of catecholamines in *S.* Typhimurium motility could be the ability of Epi and NE to bind iron with different media and their sources having various concentrations of iron. NE can function as a siderophore, and Bearson et al. demonstrated that *S.* Typhimurium motility was enhanced on DMEM with exogenous addition of iron (Freestone et al. 2000; Bearson et al. 2010); maximal bacterial motility was achieved by *S.* Typhimurium in the presence of 100 μM NE and 80 μM $FeCl_3$. Due to the contentious role of *S.* Typhimurium QseC sensor kinase in catecholamine signaling, the description of gene expression in response to Epi and NE will be predominately devoted to wild-type *S.* Typhimurium.

7.2.3 Norepinephrine-Enhanced Motility

Norepinephrine-enhanced motility of *S.* Typhimurium has been demonstrated on DMEM medium containing 0.3 % agar (Bearson and Bearson 2008). Furthermore, transcriptional analysis of *S.* Typhimurium in the presence of 2 mM NE (Bearson and Bearson 2008) in serum-SAPI minimal medium using DNA microarrays and real-time RT-PCR analyses indicated that a number of flagellar and chemotaxis genes were up-regulated during NE exposure. Flagellar assembly is a complex process and requires the coordination of a cascade of early, middle and late genes for the production of gene products that ultimately result in flagellar assembly for motility (Chilcott and Hughes 2000). This hierarchy results in the amplification of the signal such that the relative level of expression is "late">"middle">"early" genes. For example, in the presence of 2 mM NE, the *S.* Typhimurium genes *fljB* (late), *fliY* (middle), *fliA* (middle) and *flhC* (early) are significantly induced 15.4-, 4.2-, 3.6-, and 1.4-fold compared to the absence of NE, respectively (Bearson and Bearson 2008). Moreira et al. also demonstrated that the transcription of *flhDC* encoding the master regulators of motility was significantly increased in LB medium in the presence of 50 μM NE compared to the absence of NE (Moreira et al. 2010). Microarray analysis by Karavolos demonstrated that *flgD* (encoding flagellar hook capping protein) was down-regulated in the presence of Epi (Karavolos et al. 2008).

7.2.4 Nutritional Immunity, Norepinephrine-Enhanced Growth, and Iron Utilization

Differential expression of iron-regulated genes of *S.* Typhimurium was demonstrated in LB medium by Karavolos et al. using microarray analysis (Karavolos et al. 2008). Specifically, *fhuAC*, *exbBD*, *entE*, *feoAB*, and *sitAB* were up-regulated and *ftn* (ferritin) was down-regulated in the presence of 50 μM Epi.

Norepinephrine-enhanced growth of *S.* Typhimurium in serum-SAPI minimal medium is due to the siderophore-like activity of NE in the presence of transferrin (Freestone et al. 1999). Bacteria require optimal concentrations of iron for growth and either excessive or insufficient quantities of iron can be toxic or inhibitory to bacteria, respectively. The ability of serum derived transferrin to sequester iron from the bacterial cell creates an iron-deplete environment resulting in no or slow growth depending on the concentration of bacterial cells. Transcriptional analysis using microarrays to monitor gene expression of *S.* Typhimurium grown in serum-SAPI medium containing 2 mM NE compared to the absence of NE confirms the iron-deplete environment of serum-SAPI medium since transcription of genes encoding iron uptake and utilization pathways are decreased in the presence of NE (Bearson et al. 2008). This indicates that NE scavenges iron from the environment in a siderophore-like manner and increases iron availability to the bacterial cell, resulting in NE-enhanced growth of *S.* Typhimurium and a decreased need for the

production of iron uptake and utilization proteins. Interestingly, although the expression of genes encoding iron acquisition proteins is down-regulated in the presence of NE, a subset of these iron uptake proteins are required for NE-enhanced growth of *S.* Typhimurium (Williams et al. 2006; Bearson et al. 2008). This suggests that the relative expression of iron acquisition genes is lower in the presence of NE but these genes are not in a transcriptional "off" state. In addition, the microarray experiments were performed during the exponential phase ($O.D._{600}=0.4$) of bacterial growth when enterochelin, salmochelin and their breakdown products are accumulating in the growth medium. Since enterochelin/salmochelin production is necessary for NE-enhanced growth of *S.* Typhimurium (Bearson et al. 2008), the accumulation of these siderophores in the presence of NE increases iron availability which concomitantly reduces the expression of genes encoding iron acquisition proteins via the iron regulator Fur.

Norepinephrine-enhanced growth in serum-SAPI minimal medium is a model of in vivo growth during systemic infection of the mammalian host. Nutritional immunity is used by the host to limit iron availability and suppress bacterial pathogen growth (Hood and Skaar 2012). As previously described, transferrin present in serum sequesters iron from the bacterial cell and prevents bacterial growth (Fig. 7.1). However, NE can assist the bacterial cell by providing iron in the presence of enterochelin/salmochelin (Burton et al. 2002; Freestone et al. 2000; Freestone et al. 2003). A countermeasure is deployed by the host immune system using siderocalin (lipocalin 2) to sequester enterochelin (Goetz et al. 2002). *S.* Typhimurium's defense is the synthesis of salmochelin via glucosylation of enterochelin which prevents binding to siderocalin (Fischbach et al. 2006; Smith 2007; Hantke et al. 2003). In addition to the binding by siderocalin, enterochelin has a high membrane affinity which results in membrane sequestration (Luo et al. 2006). Glucosylation of enterochelin to salmochelin by IroB and hydrolysis of salmochelin by the periplasmic hydrolase IroE decreases membrane affinity and increases the iron acquisition rate for *S.* Typhimurium. Therefore the ability of *S.* Typhimurium to produce, transport and breakdown salmochelin via products of the *iroA* gene cluster is a virulence hallmark that assists *S.* Typhimurium in causing systemic disease (Fischbach et al. 2005; Smith 2007; Luo et al. 2006). This is in contrast to most *E. coli* strains, except a subset that also contain the *iroA* gene cluster including uropathogenic *E. coli* (Hantke et al. 2003).

The ability of NE to enhance the growth of *S.* Typhimurium in serum-SAPI minimal medium (Freestone et al. 1999; Williams et al. 2006; Bearson et al. 2008) suggests that additional biosynthetic pathways would be modulated besides the iron utilization and transport genes. Transcriptional analysis using microarrays on *S.* Typhimurium grown in serum-SAPI medium containing 2 mM NE revealed that NE exposure increases transcription of genes involved in amino acid biosynthesis, cofactor biosynthesis, central intermediary metabolism, energy metabolism, and synthesis of transport and binding proteins (Bearson et al. 2008). Thus, to take advantage of the increased availability of iron provided by NE in serum-SAPI minimal medium, *S.* Typhimurium modulates the biosynthesis of multiple cellular pathways to increase growth rate.

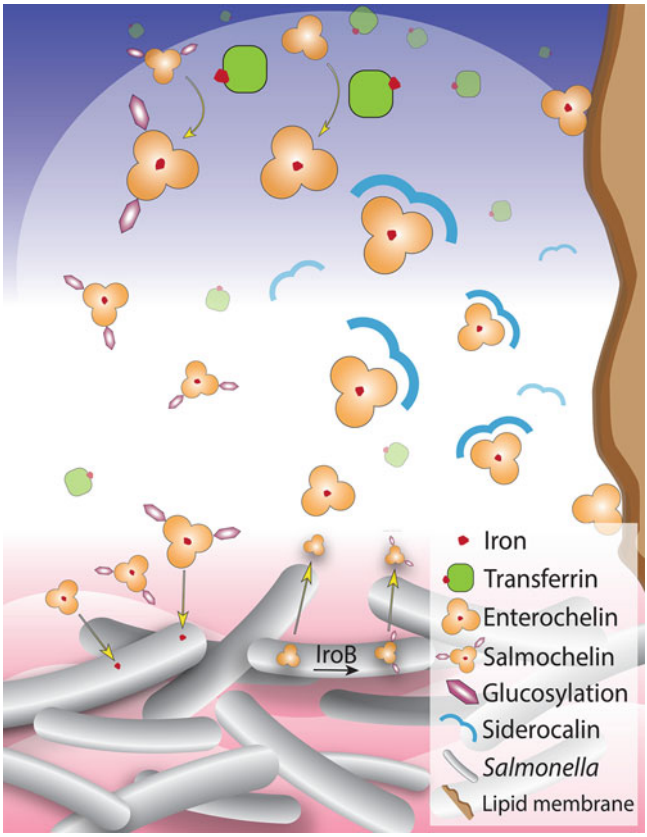

Fig. 7.1 Nutritional Immunity: a competition between mammalian iron sequestration and bacterial iron acquisition that influences bacterial growth proficiency in the mammalian host. Transferrin and other host iron binding proteins sequester iron, thereby preventing growth of microorganisms at systemic locations. Members of the *Enterobacteriaceae* family including *Salmonella enterica* produce enterochelin, a siderophore with a high affinity for iron. The bioavailability of enterochelin for bacterial iron acquisition is reduced by the mammalian siderocalin as well as the affinity of enterochelin for lipid membranes. *Salmonella* glucosylation of enterochelin to salmochelin by IroB reduces both membrane affinity and siderocalin binding. The increased bioavailability of salmochelin compared to enterochelin facilitates iron acquisition from transferrin to support pathogen growth at systemic sites. Epinephrine/norepinephrine are not required for bacterial iron acquisition in vivo. However, due to their siderophore-like properties, Epi/NE accelerate bacterial iron acquisition and therefore when present can enhance pathogen growth in iron-limited environments. (Illustration by Michael Marti)

7.2.5 SPI-1 and Invasion

Genes encoded in *Salmonella* pathogenicity island 1 (SPI-1) are important for invasion of eukaryotic cells. Moreira and Sperandio demonstrated a 1.5-fold increase in the invasion of HeLa cells by wild-type *S.* Typhimurium in the presence of 50 μM

Epi compared to the absence of the catecholamine (Moreira and Sperandio 2012). For quantitative real-time RT-PCR, cultures were grown aerobically to late log phase ($O.D._{600} = 1.0$) in LB medium with or without 50 μM NE or Epi (Moreira et al. 2010; Moreira and Sperandio 2012). Transcription of *invF* and *sopB* encoded in SPI-1 were increased >15-fold in LB medium in the presence of 50 μM NE compared to the absence of NE, whereas 50 μM Epi increased *sopB* and *sipA* transcription ~twofold in LB compared to the absence of Epi. In contrast to Moreira and Sperandio, the microarray analysis by Karavolos et al. following 30 min of exposure to 50 μM Epi (adrenaline) in LB medium during late log phase ($O.D._{600} \sim 1.0$) indicated that *invF* (encoding an invasion protein) was down-regulated (Karavolos et al. 2008). Pullinger et al. using a *prgH-gfp* reporter strain in serum-SAPI medium with and without 5 mM NE indicated that the transcription of *prgH* was low and not differentially expressed (Pullinger et al. 2010a).

7.2.6 Salmonella *Pathogenicity Island 2 (SPI-2) and SPI-2 Effectors*

Intracellular survival of *Salmonella* in epithelial cells and macrophages requires genes encoded within *Salmonella* pathogenicity island 2 (SPI-2) and additional SPI-2 effectors located outside of SPI-2 (Figueira and Holden 2012). Transcription of *ssaG* from a *gfp* transcriptional fusion was significantly reduced in *S.* Typhimurium cultures grown 16 h in serum-SAPI medium containing 5 mM NE compared to medium without NE (Pullinger et al. 2010a). The authors also demonstrated a reduction in *ssaG* expression in the presence of 25 μM Fe (III) and noted that this reduction was similar to the effect of NE. Growth of wild-type *S.* Typhimurium in LB medium to late log in the presence of 50 μM NE enhanced transcription of *sifA* greater than fivefold compared to LB medium in the absence of NE (Moreira et al. 2010; Rasko et al. 2008). A similar threefold induction of transcription was seen for cultures grown to late log in N-minimal medium pH 4.5 in response to 50 μM Epi exposure (Moreira and Sperandio 2012).

7.2.7 *PmrAB Regulon*

Members of the PmrAB regulon (*pmrFGHIJ*) were down-regulated in the presence of Epi based on microarray analysis (Karavolos et al. 2008). The PmrAB two-component signal transduction system is important for *Salmonella* resistance to antimicrobial peptides and virulence (Gunn 2008). Extracellular iron has been shown to activate the PmrAB regulon for prevention of iron toxicity (Wosten et al. 2000). The decreased expression of the PmrAB regulon may be due to sequestration of extracellular iron by Epi which would decrease iron binding and signaling by the PmrB sensor kinase.

7.2.8 *Epi- and NE-Regulated Genes Identified by Transposon Mutagenesis*

A screen of 10,000 *S.* Typhimurium Mu*dJ* transposon mutants identified seven fusions down-regulated and one fusion up-regulated in the presence of ~250 μM Epi (Spencer et al. 2010). The down-regulated genes included *virK*, *mig14*, *iroC* (see NE-enhanced growth above), *accC*, *nrdF*, *yedP*, and STM3081; the *yhaK* gene was up-regulated. Analysis of transcriptional activity using β-galactosidase assays following a 30 min exposure during mid-log phase to either 50 μM Epi or NE was confirmed in M9 minimal medium for down-regulated genes and LB medium for the up-regulated gene, *yhaK*. The regulation of *yhaK*, *virK*, and *mig14* by 500 μM Epi and NE could be reversed in a promoter-luciferase fusion assay by addition of 500 μM phentolamine, an α-adrenergic antagonist. Both *virK* and *mig14* are *Salmonella* virulence genes involved in bacterial resistance to antimicrobial peptides (Brodsky et al. 2002; Detweiler et al. 2003). Exposure to 500 μM Epi or NE significantly increased sensitivity of wild-type *S.* Typhimurium to the antimicrobial peptide cathelicidin LL-37 (Spencer et al. 2010). Furthermore, a significant increase in sensitivity to LL-37 was demonstrated for the *virK* mutant compared to wild-type *S.* Typhimurium in the absence of catecholamines.

7.2.9 *Horizontal Gene Transfer*

Exposure to 5 μM NE in LB medium significantly increased conjugation frequency of a plasmid encoding multidrug resistance from a donor *S.* Typhimurium strain to a recipient *E. coli* strain (Peterson et al. 2011). Enhanced plasmid transfer was associated with a significant up-regulation of *tra* gene expression involved with plasmid transfer; specifically, the transcription of *traGIJRY* was increased upon exposure to NE. Treatment with 500 μM phentolamine reduced the NE-enhanced conjugation frequency to baseline levels as did propranolol but this effect was delayed. Treatment with adrenergic antagonists did not reduce the baseline conjugation frequency of the *S.* Typhimurium donor. This study suggests that bacterial exposure to catecholamines may influence the evolution and adaptation of pathogens in the environment due to the transfer of genes that encode resistance to antibiotics and virulence factors.

7.3 *Escherichia coli*

7.3.1 *Experimental Conditions*

Investigations of *E. coli* exposure to catecholamines have also used various media and concentrations of catecholamines. The experimental conditions for DNA microarray analysis by Dowd involved a 1:50 dilution of an *E. coli* O157:H7 EDL933

overnight culture in serum-SAPI medium with or without 50 μM NE (Dowd 2007). The EDL933 culture was harvested following incubation for 5 h at 37 °C, 0.05 % CO_2, 95 % humidity. Bansal et al. also utilized EDL933 to analyze biofilm gene expression using microarrays in the presence of 50 μM Epi, NE, or untreated controls (Bansal et al. 2007). The EDL933 biofilms were developed for 7 h on 10 g of glass wool in 250 ml LB, 0.2 % glucose with a starting bacterial turbidity of ~0.03. DNA microarrays were employed to transcriptionally analyze *E. coli* O157:H7 86-24 harvested for RNA extraction at $O.D._{600} = 1.0$ following growth in low-glucose Dulbecco's modified Eagle's medium (DMEM) with and without 50 μM Epi at 37 °C with shaking at 250 rpm (Njoroge and Sperandio 2012).

7.3.2 Motility

Bacterial motility in the presence of Epi or NE is one of the most often investigated phenotypes of *E. coli* in response to catecholamines. Using an agarose plug chemotaxis assay, both Epi and NE were chemoattractants in a concentration-dependent migration of EDL933 towards the catecholamines (Bansal et al. 2007). Furthermore, Epi and NE increased motility 1.4-fold compared to the control culture on motility medium containing 1 % tryptone and 0.25 % NaCl. Microarray analysis of EDL933 biofilm cultures in the presence of Epi and NE by Bansal et al. demonstrated a significant increase in *fliD* encoding a flagellar hook-associated protein but a decrease in *motB* encoding a subunit for the flagellar proton motive force generator. Transcription of *fliC* encoding a flagellin protein was significantly increased approximately twofold in the presence of 50 μM Epi in DMEM compared to cultures without catecholamines (Rasko et al. 2008). The presence of 50 μM NE has been shown in multiple investigations to significantly enhance the motility of *E. coli* O157:H7 86-24 in DMEM motility assays (Sharma and Casey 2014a, b). However, an increase in the transcription of genes in the flagellar and chemotaxis operons is not always apparent, probably due to the experimental conditions used. Gene expression assays are usually performed using broth cultures whereas motility assays are typically performed using semi-solid agar medium. The differences in incubation conditions including growth rate and growth phase for broth and motility assays may account for a lack of congruence between transcriptional analysis and motility phenotype.

7.3.3 The QseBC Two-Component System

The role of regulatory proteins in catecholamine enhanced phenotypes including motility is an area of intense research. The QseC sensor kinase has been proposed to be a bacterial adrenergic receptor (Clarke et al. 2006). Multiple investigations using EHEC and UPEC isolates have demonstrated that *qseC* mutants have decreased motility compared to wild-type *E. coli* (Sperandio et al. 2003; Hughes et al. 2009;

Kostakioti et al. 2009; Hadjifrangiskou et al. 2011; Guckes et al. 2013). Inactivation of the *qseC* gene in the presence of an active QseB response regulator results in pleiotropic effects including virulence attenuation, metabolic dysregulation and decreased motility (Kostakioti et al. 2009; Hadjifrangiskou et al. 2011). Multiple physiological pathways are perturbed in *qseC* mutants, including a compromised TCA cycle. Interrogation of the TCA cycle in *E. coli* UPEC revealed that Δ*sdhB* and Δ*mdh* mutations confer virulence attenuation and decreased motility compared to the wild-type strain (Hadjifrangiskou et al. 2011); these phenotypes are similar to those of a *qseC* mutant, indicating that the presence of an active QseB in the absence of QseC results in decreased motility and metabolic dysregulation. In support of this hypothesis, *qseB* mutants of both EHEC and UPEC do not have decreased motility or virulence attenuation compared to wild-type *E. coli* (Hughes et al. 2009; Kostakioti et al. 2009). Furthermore, Sharma and Casey demonstrated that an *E. coli* O157:H7 *qseBC* mutant has a similar motility phenotype on DMEM motility medium compared to wild-type O157:H7 (Sharma and Casey 2014b). However, in response to 50 μM NE, Sharma and Casey demonstrated a significant increase in the motility of *E. coli* O157:H7 *qseC* and *qseBC* mutants (Sharma and Casey 2014a, b). These results suggest that either QseC is not a sensor for catecholamines or as has been suggested, multiple regulatory systems sense and response to Epi and NE in *E. coli* (Njoroge and Sperandio 2012; Karavolos et al. 2013). Due to the pleiotropic effects displayed by a *qseC* mutant, a clear consensus concerning the role of specific two-component systems in catecholamine sensing is lacking. For this reason, differential expression of genes regulated by the QseBC and other two-component systems in response to catecholamines will not be further discussed for *E. coli*. Instead, readers are referred to publications by Hughes et al. and Njoroge and Sperandio for further information (Hughes et al. 2009; Njoroge and Sperandio 2012).

7.3.4 E. coli *O157:H7 Locus of Enterocyte Effacement (LEE)*

The locus of enterocyte effacement (LEE) is a pathogenicity island that contains multiple operons and is present in enteropathogenic (EPEC) and various enterohemorrhagic *E. coli* (EHEC). As shown by quantitative RT-PCR, exposure of EHEC strain 86-24 to 50 μM Epi increased the expression of the *ler* gene encoding a regulator of LEE expression by ~1.5-fold (Rasko et al. 2008). Microarray analysis and quantitative RT-PCR by Dowd demonstrated that the most highly induced genes in EDL933 due to 50 μM NE exposure were *espAB* encoded in the LEE4 operon (Dowd 2007). The *eae* gene in the TIR operon was also highly expressed in the presence of NE. Using microarrays Njoroge and Sperandio indicated that exposure of 86-24 to 50 μM Epi increased the expression of LEE genes and non-LEE encoded virulence effectors (Njoroge and Sperandio 2012). Quantitative RT-PCR confirmed that expression of the LEE effector *espA* and the non-LEE effector *nleA* were increased two- and sixfold in the presence of Epi, respectively.

7.3.5 Shiga Toxin and the SOS Response

The shiga toxins Stx1 and Stx2 are encoded within lambdoid prophages integrated into the chromosome of *E. coli* O157:H7 strains. Similar to other phage-encoded genes, the regulation of *stx1* and *stx2* may be stimulated by environmental stresses that induce an SOS response. Microarray analysis by Dowd in serum-SAPI medium demonstrated that transcription of *stx1*, *stx2, umuD*, *recB*, and several phage-encoded gene products (endolysins and holin) were increased in EDL933 due to 50 μM NE. Induction of the SOS response is typically due to environmental stresses that induce DNA damage and the concomitant response to repair damage to nucleic acids. One possible explanation for induction of DNA damage is the enhanced bioavailability of iron due to NE with elevated intracellular iron increasing the vulnerability of bacterial DNA to oxidative damage (Touati et al. 1995). Dowd also suggested that NE could be an inducer of a positive adaptive state.

7.3.6 Iron Acquisition

Regulation of iron uptake and utilization genes is a common theme following exposure to catecholamines, and microarray analysis by both Dowd and Bansal et al. confirmed this effect (Dowd 2007; Bansal et al. 2007). Exposure of biofilms by Bansal et al. to 50 μM Epi or NE induced *feoAB*, *fhuBCD*, and additional iron regulated genes. Cultures of EDL933 grown in serum-SAPI medium in the presence of 50 μM NE increased expression of *fecCD*, *fhuD*, and *feoB*; other iron-regulated genes were down-regulated by NE including *fepAC*, *entCD*, and the ferric uptake regulator *fur*. The results of Dowd are consistent with the iron deplete conditions of serum-SAPI medium due to transferrin present in mammalian serum that sequesters iron from the bacterial cell in opposition to the property of NE to bind iron and promote the growth of *E. coli*.

7.3.7 Cold Shock

Investigations by both Bansal et al. and Dowd found that genes encoding cold shock proteins were induced due to exposure to 50 μM Epi and NE (Bansal et al. 2007; Dowd 2007). Bansal et al. demonstrated that the expression of the cold shock regulator genes *cspGH* were increased 6- to 23-fold due to the presence of Epi or NE. In addition, the cold shock genes *cspE* and *deaD* were also up-regulated due to catecholamine exposure. Dowd demonstrated that the expression of *cspG* and *cspH* increased 3.4- and 3.6-fold in the presence of NE, respectively.

7.4 Prospective Research

Investigation of microbial endocrinology is still in its infancy with fundamental research having demonstrated that microorganisms, including certain pathogens, can sense and respond to mammalian hormones. Most of the research investigating microbial endocrinology has been performed in vitro utilizing single hormones and analysis of individual bacterial phenotypes as a surrogate for complex environments found in nature and the mammalian host. Therefore, our current understanding of the bacterial phenotypes associated with microbial endocrinology tend to be one dimensional with a lack of knowledge concerning phenotypic networks that are expressed in response to a hormone signal. For the few investigations that have analyzed global gene expression patterns, our understanding of the interaction between bacterial regulons and stimulons is obscured by a list of genes with unknown functions. A future challenge is to understand the response of enteric bacteria to multiple, concurrent signals including hormones and environmental signals that may influence gene expression cooperatively or antagonistically. This will require additional knowledge concerning signals in a given environmental niche and the elucidation of roles for differentially expressed genes of unknown function. An example is provided below of the requirement for additional information in order to enhance our understanding and integrate multiple signals in a complex environment.

Swine are often asymptomatically colonized with *Salmonella,* and it has been known for greater than 40 years that stress (transportation, feed withdrawal, social, etc.) increases *Salmonella* fecal shedding in pigs colonized with the pathogen (Williams and Newell 1970). Recently, two investigations have demonstrated that mammalian stress hormones are involved in *Salmonella* recrudescence and can increase fecal shedding in swine. Pullinger et al. demonstrated that 6-hydroxydopamine (6-OHDA) administration to swine increased fecal shedding of pigs colonized with *Salmonella* in a porcine model of colonization (Pullinger et al. 2010b). Administration of 6-OHD, a selective neurotoxin, releases NE into the gastrointestinal tract by destroying noradrenergic nerve terminals. Increased *Salmonella* shedding in swine following 6-OHD administration suggests that norepinephrine release during animal stress increases *Salmonella* recrudescence/shedding in animals already colonized with the pathogen. Using a feed withdrawal stress model for *Salmonella*-colonized swine, Verbrugghe et al. demonstrated that stress increases serum cortisol levels with an associated recrudescence in *Salmonella* shedding (Verbrugghe et al. 2011). Also, exposure of alveolar macrophages in vitro to cortisol increased *Salmonella* intracellular proliferation. The increased bacterial proliferation did not occur following exposure of alveolar macrophages to NE or dopamine. In addition, the increased *Salmonella* proliferation in alveolar macrophages in response to cortisol was not associated with direct exposure of *Salmonella* to the hormone, and microarray analysis indicated that bacterial genes were not differentially expressed in response to cortisol exposure. The studies by Pullinger et al. and Verbrugghe et al. indicate that multiple mammalian hormones, including Epi, NE, and cortisol, may stimulate *Salmonella* recrudescence in swine. These and potentially other hormones or host

factors (such as cytokines) may have both direct and indirect effects on host cells, pathogens, and the host microbiota to influence the magnitude and duration of pathogen colonization and shedding. To date, investigations of microbial endocrinology have focused on an individual hormone (such as NE or cortisol) and an individual phenotype (such as motility or recrudescence). An integrated understanding of the host/bacterium relationship in the context of the complex host has yet to be realized. Tissue specific investigations of the host/pathogen response to microbial endocrinology are currently possible (for example, utilizing RNAscope), but a lack of knowledge concerning the most appropriate host tissues and potential genetic pathways to target for analysis limit research progress. With technological advances in nucleotide sequencing, imaging, proteomics, and metabolomics, future investigations should be capable of interrogating multiple interactions that influence both eukaryotic and prokaryotic cells that culminate in phenotypes that have already been described, as well as others yet to be discovered.

7.5 Concluding Remarks

Research investigations into the bacterial phenotypes induced by catecholamine exposure of *E. coli* and *Salmonella enterica* continue to emerge in conjunction with the associated gene expression profiles. To date the phenotype that is best understood is increased bioavailability of iron due to Epi-/NE-enhanced bacterial growth in serum containing medium. Multiple catecholamine sensor/regulators have been proposed by various investigators but our current knowledge concerning their relative importance is partially obscured due to pleiotropic effects. Continued research should clarify the role of catecholamine sensor/regulators in gene modulation by minimizing non-specific consequences.

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Chapter 8
Staphylococci, Catecholamine Inotropes and Hospital-Acquired Infections

Primrose P.E. Freestone, Noura Al-Dayan, and Mark Lyte

Abstract Patients in hospital intensive care units have long been recognized as being at high risk for developing infections from bacteria, fungi, and viruses from within the hospital locality. Risk factors for development of nosocomial infections have usually focussed on the patient's physical condition and the number and type of invasive medical procedures administered. Using the staphylococci as its focus, this chapter presents recent evidence that some of the medications routinely used in the treatment of acutely ill patients may also be a risk factor for the development of nosocomial infections.

8.1 Introduction: Nosocomial Infections

Patients in hospital intensive care units (ICU) are at particular risk of developing infections from bacteria, fungi, and viruses acquired from within the hospital environment; such infections are also often multispecies in their presentation (Vincent et al. 1995). Surveys on the incidence of ICU patients developing nosocomial infections during their hospital stay have produced varying figures, but it is generally agreed that over 1:5 patients will pick up a hospital-acquired infection, resulting in extended stays in intensive care beds, increased overall patient morbidity and, of course, avoidable and greater economic outlays (Vincent et al. 1995). The main patient-associated risk factors for the development of nosocomial infections are recognized as coming from lowered physical fitness due to severe prior illness,

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accidental or intentional tissue trauma such as surgery, reduction in immune competence (which can be medication-induced), or colonization by infectious microbes. Other factors recognized as leading to increased risk of infection involve invasive treatment procedures, particularly use of endotracheal tubes (with mechanical ventilation), urinary catheters, surgical drains, and intravascular catheters.

An additional factor that might also predispose acutely ill patients to development of potentially life-threatening infections has recently been recognized as coming from the medications they are given. Surveys of drug usage within ICUs indicate patients may receive up to 20 medications during their stay, with up to half of these patients receiving catecholamine inotrope (dopamine, epinephrine, norepinephrine, dobutamine, isoprenaline) support to maintain renal and cardiac function (Smythe et al. 1993). Work from our laboratories has shown that all of these drugs are able to markedly increase the growth and infectivity of pathogenic and commensal bacteria (see Chap. 3) by rendering blood and serum markedly less bacteriostatic, and by direct effects upon the bacteria themselves, such as enhanced expression of virulence factors (Freestone et al. 2008). Surveys of the types of bacteria causing the majority of nosocomial infections have identified the following as most prevalent: *Staphylococcus aureus* (30%), *Pseudomonas aeruginosa* (29%), coagulase-negative staphylococci such as *Staphylococcus epidermidis* (19%), yeasts such as *Candida* (17%), endogeneous enterics such as *Escherichia coli* (13%), Gram-positive enterococci (12%), *Acinetobacter* (9%), and *Klebsiella* (8%) (Spencer 1996, Vincent et al. 1995). Interestingly, all of the bacterial species shown are responsive to the catecholamine inotropes (Freestone et al. 1999, 2002, 2008; Freestone and Lyte 2008; Neal et al. 2001). Other chapters in this book examine catecholamine interactions with Gram-negative bacteria in some detail, and since the staphylococci are statistically the most prevalent pathogens in the ICU, they will form the focus of the remainder of this chapter.

8.2 Historical Evidence Suggesting a Role for Microbial Endocrinology in Infectious Diseases of the Acutely Ill

Catecholamine inotropes are routinely employed in the critical care setting to support heart function through their ability to increase cardiac contractility (see Chap. 3 for the structures of the catecholamine inotropes). For example, the inotropic agent, dobutamine is used in the treatment of congestive heart failure, epinephrine for the treatment of anaphylactic shock and dopamine to support renal function. Clinical evidence of the role of catecholamines, both endogenous and administered in the infectious disease process, has only been appreciated in the last decade or so, even though evidence of its existence has been around for nearly 80 years (see Chap. 1 for a fuller history of the field of microbial endocrinology and its relevance to medical practice). The ability of neuroendocrine hormones to influence the in vivo growth of pathogenic bacteria was first observed in 1930 (Renaud and Miget 1930). Prior to the advent of disposable syringes, metal needles and glass syringes were

reused constantly between patients with only a cursory cleaning in alcohol. Patient to patient transmission of infectious disease was frequently encountered due to the inadequate alcohol treatment of syringe needles, which could only marginally kill actively growing (vegetative) bacterial cells, but not bacterial spores. As is well understood today, certain vegetative bacteria such as *Clostridium perfringes*, the causative agent of gas gangrene, can undergo sporulation. Such spores, which are formed from the vegetative cells under conditions of nutritional deprivation, are totally resistant to alcohol treatment and can only be killed by autoclaving.

From the 1930s onward, reports associating the use of contaminated needles with administration of epinephrine solutions in the development of rapidly disseminating infections began to increase in number (Brocard 1940; Cooper 1946; Evans et al. 1948). A previously used syringe needle to treat a gas gangrene patient was then used to administer epinephrine to a patient for urticaria. Within 6 h, a fatal fulminating gas gangrene infection developed. These reports noting the rapidity of infectious spread in patients receiving epinephrine injections with contaminated needles led A.A. Miles in 1948 (Evans et al. 1948) to begin a series of experiments examining the role of catecholamines in bacterial pathogenesis. In these experiments, the ability of epinephrine to modulate the in vivo growth of both Gram-positive and Gram-negative bacteria in a guinea pig model was conclusively demonstrated in tissue slices with enhancement of growth of bacteria coinjected with epinephrine that was log orders greater than that for control slices coinjected with saline. It should be noted that norepinephrine was not investigated. The authors concluded that the ability of epinephrine to dramatically enhance bacterial growth was due to some protective coating of the bacteria by epinephrine or an epinephrine-induced inhibition of immune cell function. The testing of each of these possible mechanisms, however, met with failure (Evans et al. 1948). Significantly, at no time did these authors or others suggest that the action of epinephrine on bacterial growth was due to a direct, nonimmune effect as is currently proposed (Lyte 1992, 1993). These references had not been previously recognized since they occurred before 1966 and as such are not referenced by Medlars or other computer-based bibliographic retrieval services. Interestingly, one of the frequently used techniques by microbiologists to enable gas gangrene infections to "take" in mice has been the coinjection of epinephrine along with *C. perfringes*. It can reasonably be inferred that this practice dates back to the decades old reports described earlier.

A clearer realization that the catecholamine might be directly interacting with the infectious agents rather than aspects of host immunity were not made, and it took more than 20 years more before even an indirect association between plasma levels of catecholamine stress hormones and infectious disease episodes was realized. This came about when Gruchow (1979) observed that infectious disease episodes in patients had a tendency to occur after medical procedures that led to increased levels of catecholamines, while Groves et al. (1973) reported that postoperative patients who developed acute septic states had higher levels of epinephrine and norepinephrine in their blood than patients who experienced straightforward noninfected postoperative recoveries.

Catecholamine levels within the human body are tightly regulated and plasma clearance is usually rapid in the healthy, resulting in normal circulatory levels in the nanomolar range (Goldstein et al. 2003). However, catecholamine concentrations in patients receiving inotropic support can be several orders of magnitude higher. For example, dopamine is typically infused intravenously into acutely ill patients over a concentration range of 1–15 μg/kg/min (British National Formulary 2009). Dopamine has a half life of several minutes (Goldstein et al. 2003), and steady state levels in plasma vary according to infusion levels and general metabolic fitness, with acutely ill patients showing slower elimination rates. There is therefore a wide variation in dopamine plasma concentrations in patients receiving inotrope supplementation, ranging from ~50 nM (Johnston et al. 2004) to nearly 5,000 nM (5 μM) (Girbes et al. 2000). We have used in vitro analyses of bacteria-catecholamine interactions to show that μM concentrations of dopamine and norepinephrine are high enough to induce bacterial growth in serum and blood based media. Once within the circulation, dopamine also undergoes enzymatic conversion to norepinephrine, and metabolism of both leads to a range of compounds including dihydroxyphenylacetic acid (which can exist in plasma at 50 times the level of dopamine), dihydroxymandelic acid, and dihydroxyphenylglycol (Goldstein et al. 2003). In analyses of bacterial interactions with inotropic agents, we have found that dihydroxyl-containing intermediates of dopamine and norepinephrine (which include dihydroxymandelic acid, and dihydroxyphenylglycol), though pharmacologically inactive, still retain their ability to induce bacterial growth to a level comparable with the original catecholamine (Neal et al. 2001; Freestone et al. 2002). Catecholamine and metabolite effects can be additive (our unpublished data), and consideration of catecholamine-levels alone may not fully reveal the possible effects of metabolized inotropic agents on any bacteria that come into contact with them. The combinational effects of endogeneous catecholamines plus administered catecholamines plus their metabolites could result in changes in the blood that lead to the proliferation of infectious bacteria.

8.3 Staphylococcal Infections in the ICU

Most infections caused by the staphylococci are due to *S. aureus*, an often antibiotic resistant microbe that possesses a diverse array of virulence factors. However, in recent years and in correlating with increase usage of invasive medical procedures, the more opportunistic pathogen coagulase-negative staphylococci (C-NS), and in particular *S. epidermidis*, have become one of the most important causes of nosocomial infections (Huebner and Goldman 1999; Geffers et al. 2003). The C-NS constitute a major component of the skin microflora and were for many years regarded as saprophytes, or at least as organisms with no or low virulence. However recently, the C-NS, in particular *S. epidermidis*, have become recognized as serious nosocomial pathogens associated with indwelling medical devices such as catheters and prosthetic joints Surveys have shown that of the 5 million intravascular catheters

employed each year, approximately 250,000 CVC-related bloodstream infections are reported with an attributable mortality of up to 25%. The microbes most commonly isolated from indwelling medicals devices are the C-NS. The increasing incidence of infections caused by these normally commensal bacteria is largely due to their affinity for the biomaterials of the invasive technologies integral to modern medicine. In association with appropriate biomaterial surfaces, such as those ranging from polysilicone in catheters to steel in hip replacements, *S. epidermidis* and other C-NS adhere and proliferate to form biofilms, highly complex structures that represent functional communities of microbes. The site of insertion through the skin is one of the most common sources of bacterial contamination of intravenous catheters. Skin commensals are thought to migrate along the external surface of the catheter and colonize the intravascular tip eventually forming a biofilm. Bloodstream infections can occur if sections of the biofilm shear off and bacteria are flushed into the circulation. In a biofilm mode of existence, bacteria are protected against attack from both the immune system and antibiotic treatment, and infections by such organisms are particularly difficult to eradicate.

8.4 Catecholamine Inotropes Induce Staphylococcal Growth and Biofilm Formation on Intravascular Catheters

The requirement for iron in growth is recognized for the vast majority of pathogenic and commensal bacteria including the staphylococci (Ratledge and Dover 2000). Under normal conditions, the human body seeks to severely limit the availability of free iron to approximately 10^{-18} M through the production of the iron sequestering transferrin (blood) and lactoferrin (mucosal secretions and the gastrointestinal tract), to a concentration of iron that is below the level required to support the growth of bacteria. In an effort to obtain iron from host iron-binding proteins such as transferrin, bacteria have developed a variety of mechanisms including ferric reductase, transferrin and lactoferrin binding proteins, and ferric iron sequestering siderophores (Ratledge and Dover 2000). The importance of iron as a determinant of *S. epidermidis* biofilm formation has been demonstrated in studies that have employed iron restriction. For example, staphylococcal strains of *S. epidermidis* which were initially biofilm negative, were induced to form biofilms when grown in iron-starved medium (Deighton and Borland 1993), and iron limitation generally induces slime production by the staphylococci (Baldassarri et al. 2001).

Work from our laboratories has shown that catecholamine inotropic drugs may have additional side effects to those recognized pharmacologically. We have shown that they can serve as an etiological factor in the staphylococcal colonization of indwelling medical devices due to their ability to stimulate *S. epidermidis* growth and biofilm formation (Freestone et al. 1999; Neal et al. 2001; Lyte et al. 2003). The exposure of less than 100 CFU per ml of *S. epidermidis* to pharmacologically relevant concentrations of dopamine, dobutamine, and norepinephrine resulted in only 24 h of a greater than 10,000-fold increased growth in serum and blood-based media.

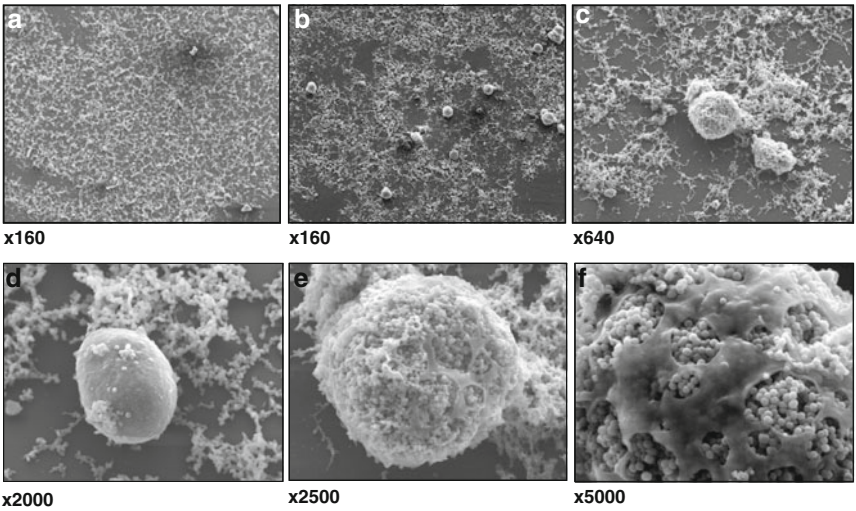

Fig. 8.1 Catecholamine-induced biofilm formation in *Staphylococcus epidermidis.* The image panels show scanning electron micrographs of biofilms of *S. epidermidis* adhering to polystyrene after overnight growth in freshly prepared plasma-SAPI medium in the absence (**a**) or presence (**b**–**f**) of 0.1 mM norepinephrine as described in Lyte et al. (2003). Initial inocula for both *S. epidermidis* cultures were approximately 10^2 CFU per ml. Higher magnification scanning electron micrographs showing details of the bacteria-exopolysaccharide clusters are shown in panels (**b**)–(**f**). This figure was taken with permission from Freestone and Lyte (2008)

The mechanism of this growth enhancement involved the drugs enabling the staphylococci access to normally inaccessible transferrin-sequestered iron (Freestone et al. 2000; Neal et al. 2001; Lyte et al. 2003; Freestone et al. 2008) (see also Chap. 3). Importantly, as well as inducing growth in normally bacteriostatic host tissue fluids, inotropes can also enhance other aspects of C-NS physiology highly relevant to their presentation in the ICU, specifically biofilm formation. This is demonstrated in Fig. 8.1, which shows a series of scanning electron micrographs of the biofilm formation that occurs when less than 100 *S. epidermidis* cells were seeded onto intravenous catheter grade polystyrene in the presence of tissue fluids they will encounter in vivo (plasma) and incubated without (a) and with (b–f) concentrations of norepinephrine that would be administered via the intravenous catheter. As is clearly visible, exposure of *S. epidermidis* to the inotrope led to massive increases in biofilm formation and production of exopolysaccharide (Lyte et al. 2003). The increasing magnification shows the mushroom like structures of bacteria and exopolysaccharide, all of which became evident in less than 48 h.

The findings demonstrated in Fig. 8.1 are clinically significant, as bacterial colonization of intravenous catheters, predominantly by the C-NS, is recognized as the most common infection encountered in the intensive care setting. There are also a number of methodological issues in the data shown in Fig. 8.1 that are worth noting. In demonstrating catecholamine inotrope induction of *S. epidermidis* biofilm,

we attempted to make the analytical conditions close to those likely to be occurring in the clinical scenario. We therefore used two methodological aspects that were markedly different from the previous studies of bacterial biofilm formation. Only 10–100 *S. epidermidis* cells were used to seed the polystyrene plastic section on which the biofilm was to be established, a low inoculum chosen to reflect the number of bacteria likely to be encountered at the beginning of the catheter colonisation. This is in contrast to many prior biofilm studies which have used several log orders higher levels of bacteria to establish biofilms. The second important difference was the use of culture conditions that employed a plasma supplemented minimal media. This again differs from studies which have used rich microbiological media to study biofilm formation, and in so doing, it may be argued, do not realistically reflect in vivo conditions in which host factors present in plasma, which are recognized to play a role in initial bacterial adhesion, are not present.

8.5 Catecholamine Inotropes Can Resuscitate Antibiotic Damaged Staphylococci

Intravenous catheter-related bloodstream infections are invariably associated with increases in length of time in intensive care units and of course hospital costs (Crnich and Maki 2001). In an effort to combat bacterial colonization of catheter lines and any possible subsequent progression to catheter-related bloodstream infections, the use of antimicrobial impregnated catheters, particularly those incorporating antibiotics such as rifampin and minocycline, is becoming increasingly adopted in the clinical care setting, However, there is a measure of doubt concerning the real efficacy of antibiotic-impregnated plastics in the prevention of catheter-related blood stream infections (Crnich and Maki 2004; McConnell et al. 2003). Concern exists as to whether they may also contribute to the emergence of antibiotic-resistant nosocomial pathogens (Sampath et al. 2001). A recent study from our laboratory might explain why antibiotics impregnation of the polymers used to construct intravenous catheter lines has not proven to be as effective as anticipated.

In this investigation (Freestone et al. 2008), we undertook two methodological approaches using norepinephrine and dopamine to investigate whether these inotropes were capable of facilitating the recovery and growth of antibiotic-damaged staphylococci (*S. epidermidis*, *S. haemolyticus* and two *S. aureus* strains, Newman and 832-4). We employed the antibiotics rifampin and minocycline as our test antimicrobials, as they are the two most frequently employed antibiotics used to coat catheters, and carried out the analyses in a serum-based culture medium (serum-SAPI) that more closely approaches the environment the bacteria would have experienced in vivo. The first experimental approach used a minimum inhibitory concentration (MIC) assay, in which the staphylococci were incubated with norepinephrine and dopamine in the presence of increasing concentrations of rifampin or minocycline (up to 100 times the MIC for each antibiotic). Representative data for rifampin is shown in Fig. 8.2 for *S. epidermidis* (a) and *S. aureus* strain Newman (b).

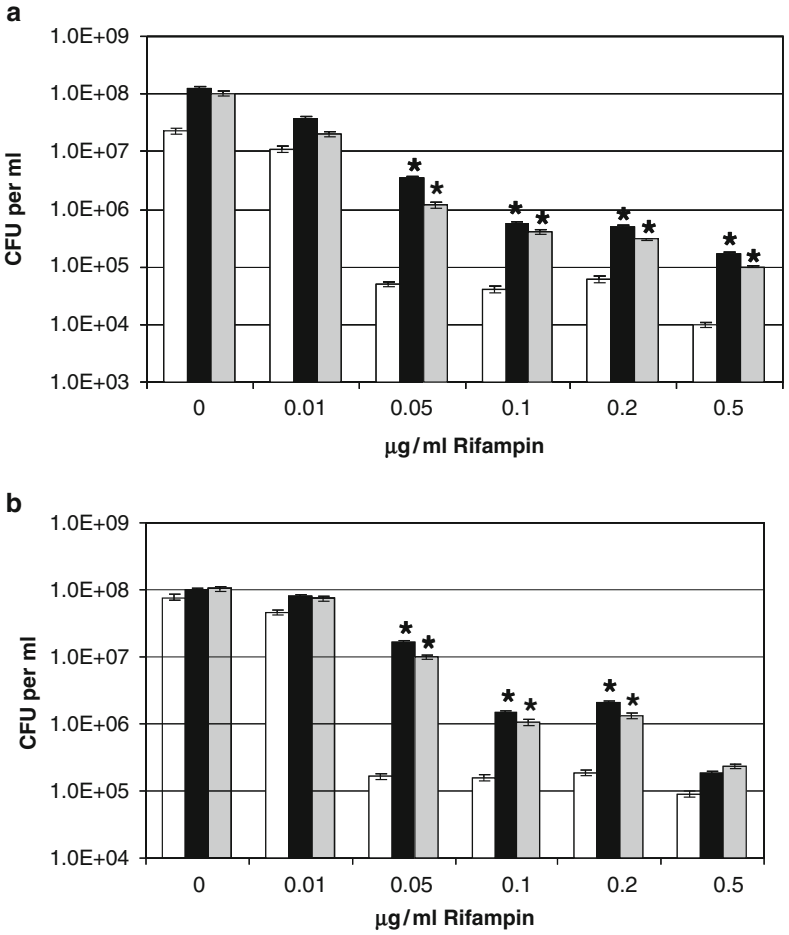

Fig. 8.2 Staphylococcal response to increasing rifampin concentrations in the presence of catecholamine inotropes. Analyses of simultaneous exposure of *S. epidermidis* and *S. aureus* to antibiotics and catecholamine inotropes were performed in serum-SAPI medium. Bacteria were incubated for 24 h 37°C in a 5% CO_2 humidified incubator and measured for growth and measured for growth using plate analysis. The results shown represent the mean of triplicate cultures; standard error of the mean was not greater than 7% for all cultures shown. The asterisk, *, indicates a statistically significant increase in growth level over the corresponding non-inotrope supplemented control culture ($P<0.0001$). (**a**) Growth response of *S. epidermidis* (inoculum size 1×10^6 CFU per ml); (**b**) Growth response of *S. aureus* Newman (inoculum size 0.92×10^6 CFU per ml). *White bar*, no additions (control); *black bar*, 100 µM norepinephrine; *gray bar*, 100 µM dopamine. This figure was taken with permission from Freestone et al. (2008)

The responses for *S. haemolyticus* and *S. aureus* strain 832-4 were very similar to those shown for *S. epidermidis* and *S. aureus* Newman and so are not shown. As can seen, for both strains simultaneous administration of inotrope and rifampin allowed more than 2 logs greater growth in the presence of rifampin concentrations around the MIC (determined to be ~0.1 µg per ml), although the catecholamines

did not significantly protect the staphylococci from the inhibitory effects of higher rifampin concentrations. A similar response profile of greater growth in the presence of the inotropes was also obtained when the staphylococci were exposed to minocycline in the presence/absence of catecholamine inotropes (data not shown).

Our second experimental approach involved analyzing whether catecholamine inotropes could rescue the growth of staphylococci pretreated with antibiotics, as it is well known that following antibiotic treatment, some acute bacterial infections can become subclinical, re-activating in response to not always understood changes in the host environment. Figure 8.3 shows the ability of norepinephrine and dopamine to rescue growth of *S. epidermidis* pre-treated for 4 h with a rifampin concentration of 5 μg per ml (approximately 100 times the MIC) and then serially diluted into serum-SAPI medium containing no additions or the catecholamine inotropes and grown on for 24 h. Figure 8.3a, b show the growth profiles of control and antibiotic treated *S. epidermidis,* and reveal that the rifampin had reduced the viable count of the treated bacteria by nearly 4 log orders. However, the presence of the inotropes significantly increased the growth of *S. epidermidis* preexposed to rifampin, even when the antibiotic carryover was near to the MIC (the 10^{-2} dilution) ($P < 0.0001$).

Earlier work from our laboratories had shown that *S. aureus* and other coagulase-positive staphylococcal strains showed little growth responsiveness to catecholamines in serum-based medium (Freestone et al. 1999; Neal et al. 2001). This observation was confirmed by the responses of nonantibiotic-treated *S. aureus* strain Newman to norepinephrine and dopamine (Fig. 8.4b), which showed that growth enhancement by the inotropes was evident at only very low cell densities (~10 CFU per ml). However, antibiotic-treatment of the *S. aureus* cultures (Fig. 8.4a) caused the bacteria to become significantly more responsive to the inotropes ($P < 0.0001$). This being so, the growth profile observed over the *S. aureus* culture dilutions is different to that obtained for *S. epidermidis* (compare b of Figs. 8.3 and 8.4). This is most likely because unlike the C-NS, the coagulase positive staphylococci possess highly efficient acquisition systems for the scavenging of host sequestered iron stores, which then enables them to overcome the iron-limitation of tissue fluids such as serum and blood without aid of the catecholamines. Indeed, when we analyzed the ability of *S. epidermidis* and *S. aureus* to remove iron from ^{55}Fe-labeled transferrin, we found that the coagulase positive staphylococci were able to extract around 10 times more iron than the C-NS. Adding the inotrope enabled the coagulase positive staphylococci to obtain around 2.5-3 times more iron from the transferrin while for the C-NS, the inotrope allowed much more Fe uptake from transferrin – around 30 times more than the noninotrope supplemented controls, such that when the inotrope were present, the final levels of uptake of transferrin-complexed iron was similar between the staphylococci. Figure 8.5 shows the ability of inotropes to rescue growth of staphylococci pretreated with 2 μg per ml minocycline (~100 times MIC). Although both *S. epidermidis* and *S. aureus* recovered better from the minocycline assault, than they did from the rifampin, inclusion of norepinephrine and dopamine still induced a much greater rate of recovery.

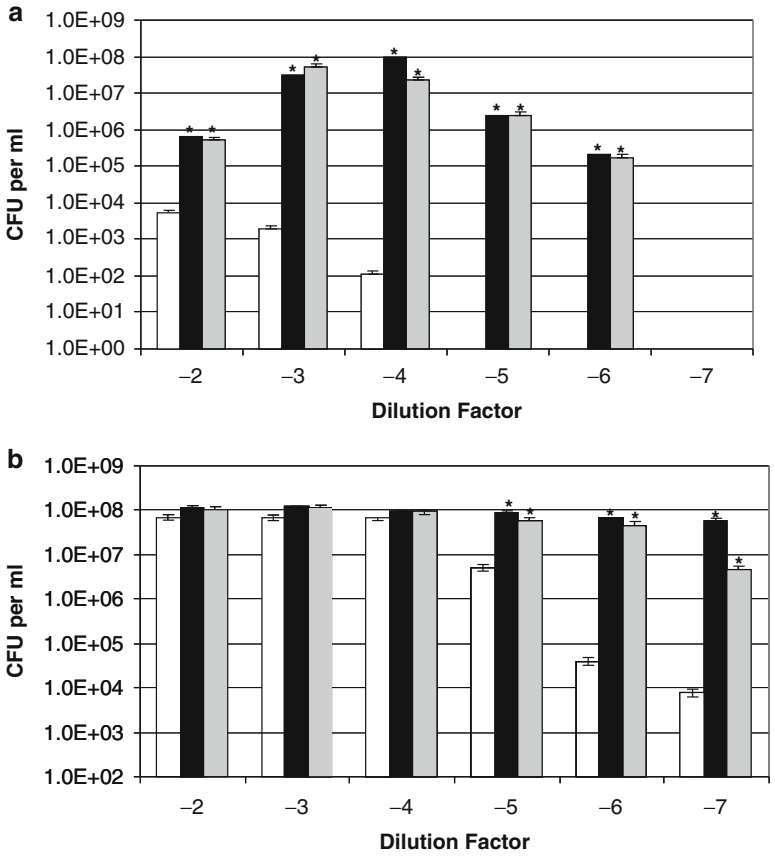

Fig. 8.3 Effect of catecholamine inotropes on coagulase-negative staphylococci pre-exposed to rifampin. Replicates of exponentially growing *S. epidermidis* cultures grown in serum-SAPI medium were incubated for 4 h at 37°C with 5 μg per ml rifampin (100 times MIC). The antibiotic-treated (inoculum size 5.40×10^4 CFU per ml) (**a**) and control cultures (inoculum size 1.05×10^8 CFU per ml) (**b**) were each then serially diluted in tenfold dilution steps into fresh serum-SAPI with no additions (*white bar*) or supplemented with norepinephrine (*black bar*) or dopamine (*grey bar*) each at a concentration of 100 μM. Test and control were incubated at 37°C for 24 h and enumerated for viable cell levels using pour-plate analysis. The asterisk, *, indicates a statistically significant increase in growth level over the corresponding non-catecholamine inotrope supplemented control culture ($P < 0.0001$). This figure was taken with permission from Freestone et al. (2008)

8.6 Fighting Back: Blockade of Staphylococcal Catecholamine Responsiveness

Previously we had shown that blockade of catecholamine growth responsiveness in enteric bacteria was possible using drugs employed therapeutically as catecholamine receptor antagonists (Freestone et al. 2007). To determine if we could similarly inhibit staphylococcal responses to the inotropes, we examined the ability of

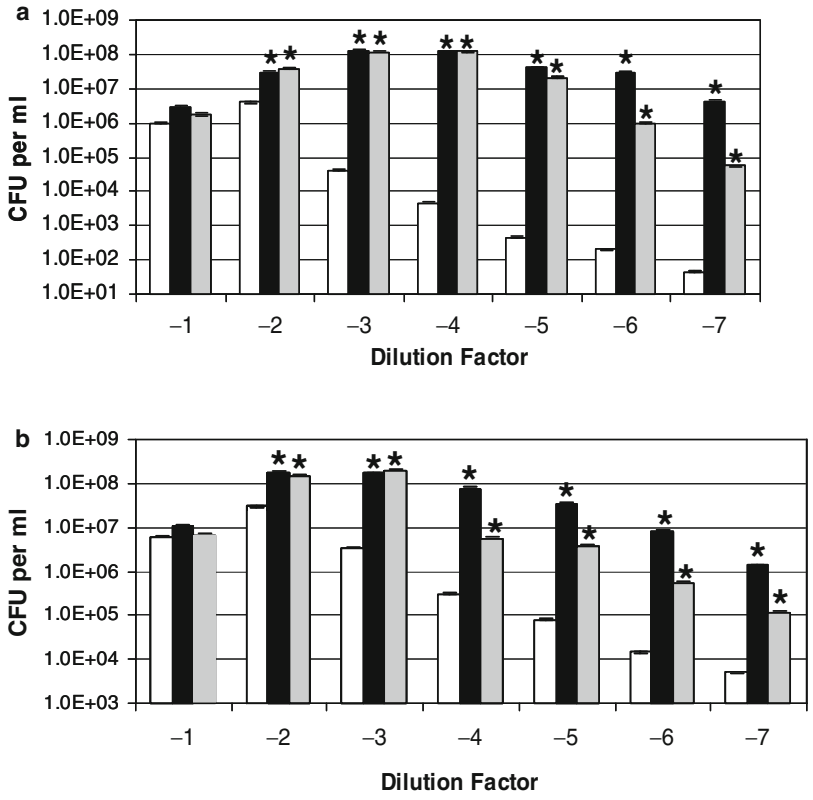

Fig. 8.4 Effect of catecholamine inotropes on coagulase-positive staphylococci pre-exposed to rifampin. Replicates of an exponentially growing *S. aureus* strain Newman cultures grown in serum-SAPI medium were incubated for 4 h at 37°C with 5 μg per ml rifampin (100 times MIC). The antibiotic-treated (inoculum size 2.21×10^5 CFU per ml) (**a**) and control cultures (inoculum size 2.22×10^8 CFU per ml) (**b**) were each then serially diluted in tenfold dilution steps into fresh serum-SAPI with no additions (*white bar*) or supplemented with norepinephrine (*black bar*) or dopamine (*grey bar*) each at a concentration of 100 μM. Test and control were incubated at 37°C for 24 h and enumerated for viable cell levels using pour-plate analysis. The asterisk, *, indicates a statistically significant increase in growth level over the corresponding non-catecholamine inotrope supplemented control culture ($P < 0.0001$). This figure was taken with permission from Freestone et al. (2008)

a range of α and β-adrenergic and dopaminergic antagonists to block *S. epidermidis* responses to norepinephrine and dopamine. As can be seen in Table 8.1, the β-adrenergic receptor antagonist propranolol had no effect on the ability of either catecholamine to stimulate growth of *S. epidermidis*. Other β-adrenergic antagonists such as labetalol, atenolol, and yohimbine also had no effect (data not shown). However, the α-adrenergic antagonists phentolamine and prazosin (data not shown) were able to inhibit growth induction by norepinephrine by over three log-orders. None of the α- or β-antagonists when tested alone induced growth of *S. epidermidis*, even at 500 μM. Furthermore, addition of Fe overcame the antagonist blockade of

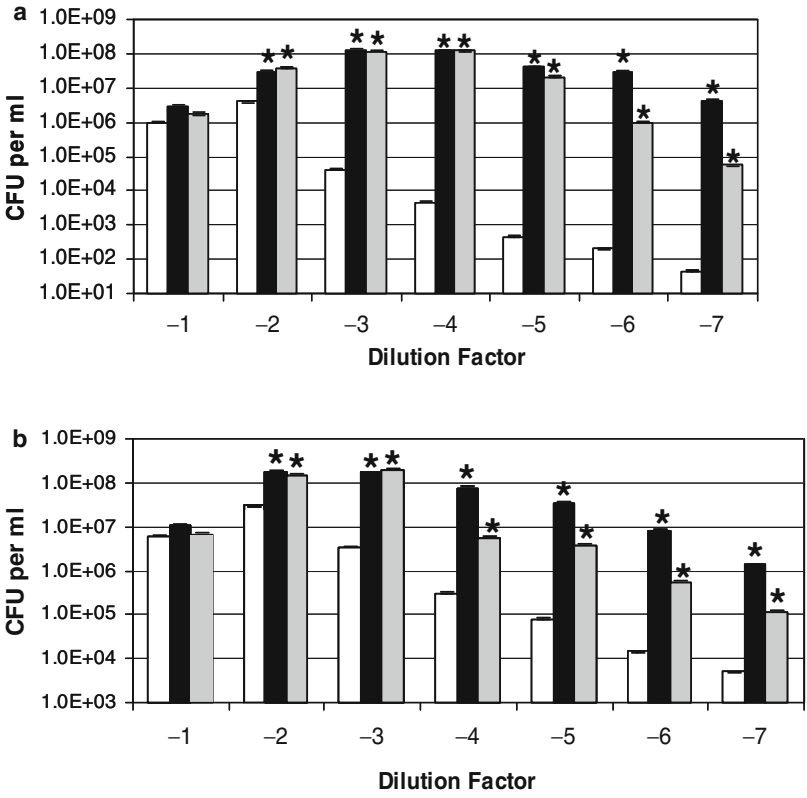

Fig. 8.5 Effect of catecholamine inotropes on staphylococci pre-exposed to minocycline. Replicates of an exponentially growing *S. epidermidis* and *S. aureus* strain Newman cultures grown in serum-SAPI medium were incubated for 4 h at 37°C with 2 μg per ml minocycline (100 times MIC). The antibiotic treated *S. epidermidis* culture (inoculum size 5.40×10^4 CFU per ml) (**a**) and *S. aureus* Newman culture (inoculum size 8.90×10^4 CFU per ml) (**b**) were each then serially diluted in tenfold dilution steps into fresh serum-SAPI with no additions (*white bar*) or supplemented with norepinephrine (*black bar*) or dopamine (*grey bar*) each at a concentration of 100 μM. The two sets of cultures were then incubated at 37°C for 24 h and enumerated for viable cell levels using pour-plate analysis. The asterisk, *, indicates a statistically significant increase in growth level over the corresponding non-catecholamine inotrope supplemented control culture ($P < 0.0001$). This figure was taken with permission from Freestone et al. (2008)

growth induction (Table 8.1), indicating that growth inhibition by the α-adrenergic receptor antagonists was not due to any cellular toxicity of the antagonist, but instead represents a specific antagonism of the staphylococcal response to the catecholamines.

The next question we asked was whether we could use phentolamine and chlorpromazine to prevent the inotrope from rescuing the growth of antibiotic-treated staphylococci. Figure 8.6 shows the effect of including the antagonists in the catecholamine resuscitation experiments described in Figs. 8.3 and 8.4. As seen in

Table 8.1 Blockade of catecholamine-induced staphylococcal growth responsiveness

		Antagonist concentration (μM)										
Antagonist	CA	0	0.1	1	10	20	50	75	100	200	300	N/A[b]
Phentolamine	NE	8.01[a]	7.94	7.86	6.90	6.08	5.15	4.90	4.60	3.75	3.75	3.90
	NE + Fe[c]	8.21	8.13	8.13	8.13	8.06	8.06	8.04	8.15	8.09	7.99	
	Dop	8.01									7.96	
Propranolol	NE	8.02	8.02	7.99	8.03	7.68	7.76	7.91	7.90	7.81	7.60	4.00
	Dop	8.01									7.95	
Chlorpromazine	Dop	8.00	7.98	7.98	7.81	7.34	6.92	6.66	6.35	5.27	4.81	4.03
	Dop + Fe[c]	8.06	8.08	8.08	8.16	8.11	8.08	8.08	8.08	8.11	8.06	
	NE	8.03									7.81	

S. epidermidis was inoculated at 10^2CFU per ml into serum-SAPI medium containing the catecholamine (CA) plus the concentrations of antagonists shown in the table, incubated statically for 24 h 37°C in a 5% CO_2 humidified incubator and measured for growth ([a]expressed as $\log^{-10}$CFU per ml) using pour plate counts. Growth levels of non-catecholamine supplemented cultures ([b]N/A) are shown for comparison purposes. Norepinephrine (NE) and dopamine (Dop) were both used at 50 μM and Fe ($Fe(NO_3)_3$)[c] at 100 μM. Results shown are representative data from at least three separate experiments; all data points showed variation of no more than 5%Data table was taken with permission from Freestone et al. (2008) Note that Fe was included to show that the effects of the antagonists were not due to toxicity of the compounds.

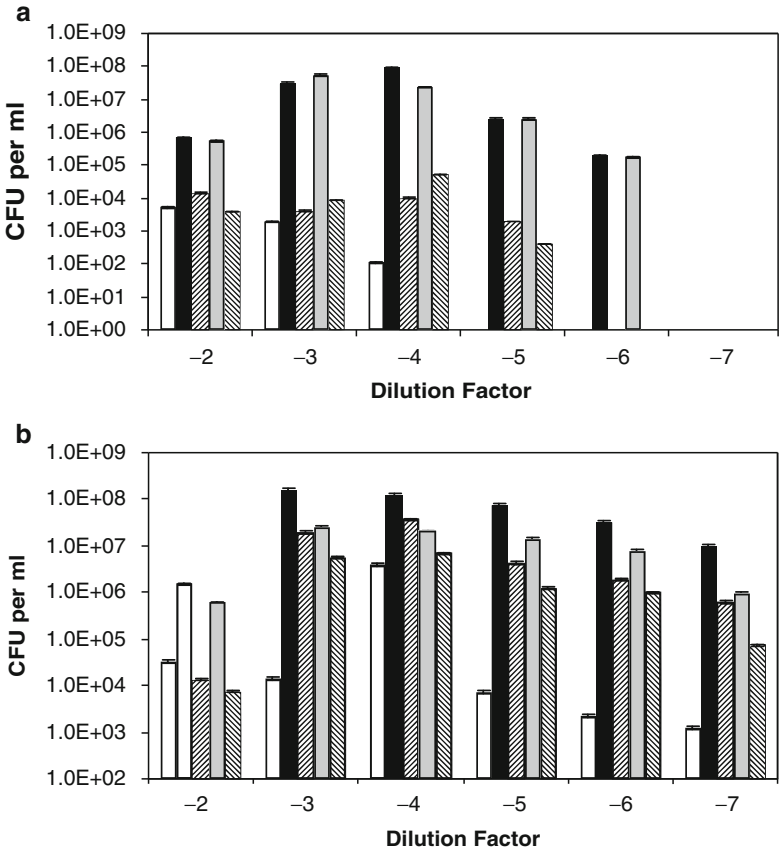

Fig. 8.6 Effects of adrenergic and dopaminergic antagonists on the ability of catecholamines to rescue of growth of antibiotic-stressed staphylococci. Replicates of exponentially growing cultures of the bacteria shown were cultured in serum-SAPI medium and then pre-incubated with 5 μg per ml rifampin as described in the legends to Fig. 8.3, and similarly further processed except that additional catecholamine assays containing norepinephrine were supplemented with 200 μM phentolamine and dopamine supplemented with 200 μM chlorpromazine. Test and non-catecholamine inotrope supplemented controls were incubated at 37°C for 24 h, and enumerated for viable cell levels using pour-plate analysis. Viable counts of the inoculating antibiotic treated *S. epidermidis*, and *S. aureus* Newman cultures were 2.21×10^5 and 7.8×10^5 CFU per ml respectively. The values shown represent means of triplicate plate counts; standard error of the mean was not greater than 7% for all cultures shown. (**a**) *S. epidermidis*, (**b**) *S. aureus White bar* no additions (control), *black bar* 100 μM norepinephrine, *right diagonal hatch* 100 μM norepinephrine plus 200 μM phentolamine, *gray bar* 100 μM dopamine, *left diagonal hatch* 100 μM dopamine plus 200 μM chlorpromazine. This figure was taken with permission from Freestone et al. (2008)

Fig. 8.6, the α-adrenergic and dopaminergic antagonists were able to specifically block norepinephrine and dopamine resuscitation of rifampin-damaged *S. epidermidis* and *S. aureus*. Further work showed that the inotrope blocking activity of a single addition of the antagonists was retained for at least 3 days for both strains (data not shown). For *S. aureus* (Fig. 8.6b), the antagonists were less potent, and at best phentolamine and chlorpromazine were able to reduce norepinephrine and dopamine resuscitation by at most a log order in magnitude.

8.7 Future Thoughts: Side Effects and Covert Side Effects

Until recently, side effects of drugs used in clinical practice have been evaluated in a somewhat limited way when considering their effects upon microorganisms. This perspective may have to change, as the evidence increases that certain nonantibiotic compounds, the catecholamine inotropes principally but also other administered drugs such as steroids and opioids may directly increase bacterial infectivity (Neal et al. 2001; Zaborina et al. 2007). Humans are the residence of up to 10^{14} highly diverse bacteria, fungi, viruses, and protozoa, and since the advent of microbial endocrinology, it is perhaps no longer surprising that changes in the hormonal milieu of the host do not go un-remarked by these prokaryotic coinhabitants. Singer (2007) has suggested that inotrope effects on bacteria such be viewed as 'covert side effects'. Given the potentially devastating consequences of the covert side effects of the catecholamine inotropes class of drugs, there is a need for further research into whether any of the other widely used pharmaceuticals can also affect infectivity of microbes through neuro-homormonal-mediated interactions that may play a part in nosocomial infections. A final thought is that it might become necessary is to extend the patient side effects information on drug labels to also include effects on the microbes inhabiting the patient.

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Chapter 9
Interkingdom Chemical Signaling in Enterohemorrhagic *Escherichia coli* O157:H7

Melissa M. Kendall

Abstract *Escherichia coli* is one of the most-studied species of bacteria due to its frequent incidence in diverse environments and hosts, as well as its use as a tool in molecular biology. Most *E. coli* strains are commensal, in that they colonize the host without causing disease; however, some strains of *E. coli* are pathogens and are able to cause diverse illnesses, including urinary tract infections, sepsis/meningitis, as well as intestinal disease that result in diarrhea (Kaper et al. 2004). Six categories of diarrheagenic *E. coli* are recognized, and these are classified in part based on how they interact with epithelial cells (Kaper et al. 2004). Of these, enterohemorrhagic *E. coli* O157:H7 (EHEC) is one of the most important pathogenic *E. coli* strains. EHEC causes major outbreaks of bloody diarrhea that can result in the development of fatal hemorrhagic colitis and hemolytic uremic syndrome (Karmali et al. 1983). EHEC colonizes the colon, where it forms attaching and effacing (AE) lesions on the intestinal epithelial cell. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, effacement of the microvilli and rearrangement of the underlying cytoskeleton, which results in formation of a pedestal-like structure beneath the bacterium (Jerse et al. 1990; Jarvis et al. 1995; Kenny et al. 1997). Most of the genes involved in the formation of AE lesions are encoded within a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al. 1995). The LEE contains 41 genes that are organized in five major operons (*LEE1, LEE2, LEE3, LEE5*, and *LEE4*) (Elliott et al. 1998, 1999; Mellies et al. 1999). The LEE encodes a type three secretion system (T3SS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990) and its receptor (Tir) (Kenny et al. 1997), as well as effector proteins (Kenny et al. 1996; Abe et al. 1997; McNamara and Donnenberg 1998; Elliott et al. 2001; Tu et al. 2003; Kanack et al. 2005). EHEC also encodes an arsenal of effector proteins located outside of the LEE that are important in EHEC virulence (Campellone et al. 2004; Deng et al. 2004; Garmendia et al. 2004, 2005; Gruenheid et al. 2004; Tobe et al. 2006).

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Keywords *E. coli* • Two component systems • Epinephrine • Signaling • Type three secretion systems • Shiga toxin

9.1 *Escherichia coli* O157:H7

Escherichia coli is one of the most-studied species of bacteria due to its frequent incidence in diverse environments and hosts, as well as its use as a tool in molecular biology. Most *E. coli* strains are commensal, in that they colonize the host without causing disease; however, some strains of *E. coli* are pathogens and are able to cause diverse illnesses, including urinary tract infections, sepsis/meningitis, as well as intestinal disease that result in diarrhea (Kaper et al. 2004). Six categories of diarrheagenic *E. coli* are recognized, and these are classified in part based on how they interact with epithelial cells (Kaper et al. 2004). Of these, enterohemorrhagic *E. coli* O157:H7 (EHEC) is one of the most important pathogenic *E. coli* strains. EHEC causes major outbreaks of bloody diarrhea that can result in the development of fatal hemorrhagic colitis and hemolytic uremic syndrome (Karmali et al. 1983). EHEC colonizes the colon, where it forms attaching and effacing (AE) lesions on the intestinal epithelial cell. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, effacement of the microvilli and rearrangement of the underlying cytoskeleton, which results in formation of a pedestal-like structure beneath the bacterium (Jerse et al. 1990; Jarvis et al. 1995; Kenny et al. 1997). Most of the genes involved in the formation of AE lesions are encoded within a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al. 1995). The LEE contains 41 genes that are organized in five major operons (*LEE1, LEE2, LEE3, LEE5*, and *LEE4*) (Elliott et al. 1998, 1999; Mellies et al. 1999). The LEE encodes a type three secretion system (T3SS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990) and its receptor (Tir) (Kenny et al. 1997), as well as effector proteins (Kenny et al. 1996; Abe et al. 1997; McNamara and Donnenberg 1998; Elliott et al. 2001; Tu et al. 2003; Kanack et al. 2005). EHEC also encodes an arsenal of effector proteins located outside of the LEE that are important in EHEC virulence (Campellone et al. 2004; Deng et al. 2004; Garmendia et al. 2004, 2005; Gruenheid et al. 2004; Tobe et al. 2006).

9.2 Regulation of the LEE Expression

Regulation of the LEE is complex and tightly regulated. The LEE pathogenicity island encodes genes for three regulators, Ler, GrlA, and GrlR (Mellies et al. 1999; Deng et al. 2004). Ler is encoded in *LEE1* and is a master regulator of the LEE (Mellies et al. 1999; Sperandio et al. 2000; Sánchez-SanMartín et al. 2001; Haack et al. 2003; Russell et al. 2007). Expression of Ler is directly or indirectly regulated by multiple proteins (Friedberg et al. 1999; Sperandio et al. 2002a, b; Umanski et al.

2002; Iyoda and Watanabe 2004, 2005; Sharma and Zuerner 2004; Iyoda et al. 2006; Sharp and Sperandio 2007; Kendall et al. 2010), including GrlR that represses *ler* transcription and GrlA that activates *ler* transcription (Deng et al. 2004; Barba et al. 2005; Russell et al. 2007). Moreover, expression of the LEE and LEE-associated genes is subject to further regulation at the transcriptional and posttranscriptional levels in response to diverse environmental cues, including nutrients and stress responses (Sperandio et al. 2003; Mellies et al. 2007; Bhatt et al. 2009, 2011; Lodato and Kaper 2009; Shakhnovich et al. 2009; Kendall et al. 2011, 2012; Njoroge et al. 2012; Pacheco and Sperandio 2012) as well as host hormones present in the gastrointestinal (GI) tract (Sperandio et al. 2003).

9.3 Shiga Toxin

The mortality associated with EHEC infections stems from the production and release of a potent Shiga toxin. EHEC expresses Shiga toxin in the intestine, and this inhibitor of mammalian protein synthesis is absorbed systemically and binds to receptors found in the kidneys and central nervous system, causing HUS, seizures, cerebral edema, and/or coma (Karmali et al. 1983). The genes encoding Shiga toxin are located within a lambdoid bacteriophage and are transcribed when the phage enters its lytic cycle (Neely and Friedman 1998; Neely and Friedberg 2000; Wagner et al. 2001). Disturbances in bacterial envelope, DNA replication, or protein synthesis (which are targets of conventional antibiotics) initiate an SOS response in EHEC that triggers the bacteriophage to enter the lytic cycle and produce Shiga toxin. Consequently, treatment of EHEC infections with conventional antimicrobials is contraindicated (Davis et al. 2013).

9.4 Chemical Signaling in EHEC

Bacterial pathogens rely on environmental cues derived from the host, as well as from the resident microbiota, to properly coordinate expression of traits important for pathogenesis. Quorum sensing is a cell-to-cell signaling mechanism through which bacteria synthesize and/or respond to bacterial-produced chemical signals called autoinducers (AIs). As concentrations of AI molecules change, bacteria modulate gene expression. Quorum sensing was first characterized in *Vibrio fisheri* and is based on the LuxI and LuxR proteins (Nealson et al. 1970). LuxI is a cytoplasmic protein that synthesizes the AI molecules, which then diffuses freely out of the bacterial cell. Once a particular threshold concentration of AI molecules is reached in the extracellular environment, the AI molecules diffuse back into the bacterial cells, where they interact with the transcription factor LuxR. Interaction between LuxR and its cognate AI promotes LuxR stability and oligomerization, which enables LuxR to bind target promoters and control gene expression.

EHEC relies on quorum sensing to control expression of genes encoding motility and virulence (Sperandio et al. 1999, 2001, 2002a, b, 2003). Initial studies suggested that the AI molecule called AI-2 was the signal that mediated quorum sensing-dependent virulence gene expression in EHEC (Sperandio et al. 1999, 2001); however, additional studies revealed that a distinct molecule, AI-3, was actually the signal responsible for activating expression of the LEE-encoded T3SS and motility genes (Sperandio et al. 2003). The molecule AI-2 is synthesized by a small metalloenzyme LuxS. Specifically, LuxS converts *S*-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable compound that reacts with water and cyclizes to form several different furanones (Schauder et al. 2001; Winzer et al. 2002; Sperandio et al. 2003), one of which is thought to be the precursor of AI-2 (Schauder et al. 2001). AI-3 does not directly depend upon *luxS* for synthesis; however, a mutation in the *luxS* gene affects AI-3 production by altering cellular metabolism (Walters et al. 2006). Subsequent studies that incorporated biochemical assays have conclusively demonstrated that AI-2 and AI-3 are distinct molecules. For example, the polar furanone AI-2 does not bind to C_{18} columns, whereas AI-3 binds to C_{18} columns and can only be eluted with methanol (Sperandio et al. 2003). Moreover, electrospray mass spectrometry also revealed differences between the structures of AI-2 and AI-3 (Chen et al. 2002; Sperandio et al. 2003). AI-2 activity leads to the production of bioluminescence in *V. harveyi*, and AI-3 does not show any activity for this assay. Conversely, the AI-3 activates transcription of the EHEC virulence genes, whereas AI-2 does not influence EHEC virulence. Significantly, the eukaryotic hormones epinephrine and norepinephrine (epi/NE) can substitute for AI-3 to activate EHEC virulence gene expression, including the LEE genes, and adrenergic receptor antagonists inhibit the regulatory effects of epi/NE and AI-3 (Clarke et al. 2006). Thus, although the final structure of AI-3 has not yet been elucidated, it has been hypothesized that AI-3 may be structurally similar to epi/NE (Sperandio et al. 2003) (Fig. 9.1a, b).

9.5 Infectious Disease and Hormones

Eukaryotic cell-to-cell signaling is based on a variety of hormones, which are essential for eukaryotic development and homeostasis. Significantly, the hormones epinephrine and norepinephrine also promote EHEC growth and are co-opted as signals that EHEC uses to modulate expression of virulence traits (Lyte and Ernst 1992; Lyte et al. 1996; Freestone et al. 2000; Sperandio et al. 2003). Epinephrine and norepinephrine belong to the class of hormones called catecholamines. These hormones are derived from the amino acid tyrosine and are composed of a catechol and a side-chain amine. Epinephrine and norepinephrine are the most abundant catecholamines in the human body and are involved in the fight or flight response. Epinephrine and norepinephrine are present at micromolar concentrations in the intestine (Eldrup and Richter 2000) and play important roles in physiology of the GI tract by modulating smooth muscle contraction, submucosal blood flow, and

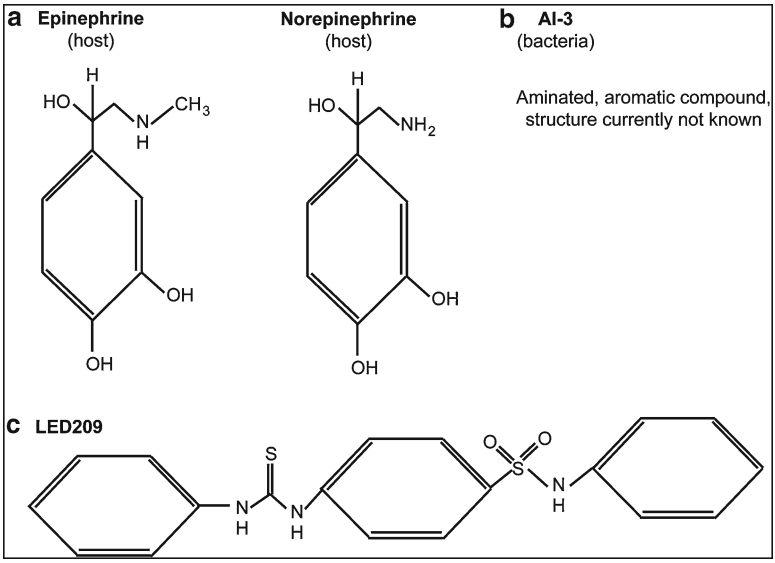

Fig. 9.1 Structures involved in adrenergic signaling. (**a**) Host hormones epinephrine and norepinephrine. (**b**) The structure of AI-3 has not been solved, but may resemble epinephrine and norepinephrine. (**c**) The structure of LED209 that inhibits QseC signaling

chloride and potassium secretion (Horger et al. 1998). In addition to the central nervous system and adrenal medulla, the adrenergic neurons that are present in the enteric nervous system are the major sources of epinephrine and norepinephrine (Furness 2000; Purves et al. 2001). Additionally, immune cells including T cells, macrophages, and neutrophils produce and secrete epinephrine and norepinephrine (Flierl et al. 2008). Therefore, bacterial infections may results in increased epinephrine and norepinephrine concentrations in the GI tract due to the stress of the infection in conjunction with the immune response. Finally, the commensal GI microbiota also contribute to the generation of biologically active norepinephrine (and to a lesser extent epinephrine) in the lumen of the GI tract (Asano et al. 2012).

9.6 Bacterial Adrenergic Receptors

The mammalian adrenergic receptors that bind epinephrine and norepinephrine and transmit signals are called G-coupled protein receptors. GPCRs are transmembrane receptors are coupled to heterotrimeric guanine-binding proteins (G proteins). EHEC does not encode G proteins; therefore, EHEC senses epinephrine and norepinephrine via a different mechanism. The main signaling transduction systems in bacteria are two component systems (TCSs) (Clarke et al. 2006). TCSs are critical for bacteria to sense and respond to changes in the environment. A typical TCS is composed of a histidine sensor kinase (HK) located in the cytoplasmic membrane that perceives a

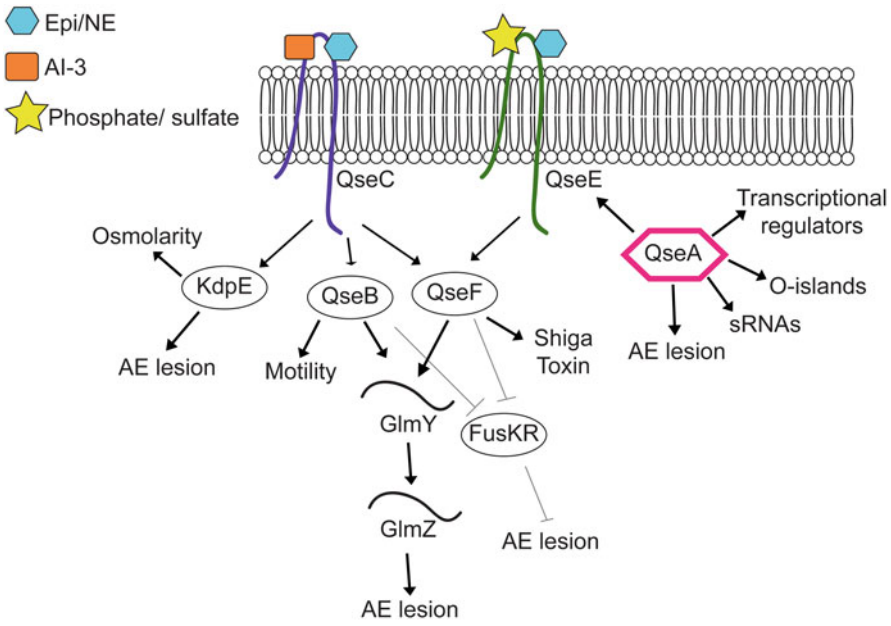

Fig. 9.2 Summary of the epinephrine/NE/AI-3 signaling cascade in EHEC. *Arrows* indicate positive regulation and *lines* with *bars* indicate negative regulation

stimulus and a cytoplasmic response regulator (RR) that controls the output (Jung et al. 2012). Upon sensing a specific environmental cue, the kinase autophosphorylates at a specific histidine residue and subsequently transfers this phosphate to an aspartate residue on its cognate RR. RRs are transcription factors that mediate the output of this signaling cascade by binding DNA to promote or repress gene expression (Jung et al. 2012). EHEC encodes two adrenergic receptors, QseC and QseE (Clarke et al. 2006; Reading et al. 2009), that upon sensing epinephrine and norepinephrine initiate a complex signaling cascade, which results in coordinated expression of virulence genes (Sperandio et al. 1999, 2002a, b, 2003; Clarke et al. 2006; Kendall et al. 2007; Reading et al. 2007, 2009; Hughes et al. 2009; Njoroge and Sperandio 2012; Pacheco et al. 2012; Gruber and Sperandio 2014) (summarized in Fig. 9.2).

9.7 The QseBC TCS

The gene encoding QseC was first identified in an array that compared gene expression between wild type (WT) EHEC and a *luxS* mutant (Sperandio et al. 2002a, b), and subsequent studies revealed that QseC directly senses, and

autophosphorylates, in response to host-derived epinephrine and norepinephrine as well as the bacterial-derived AI-3 (Clarke et al. 2006). QseC is a global regulator in EHEC and influences expression of more than 400 genes in response to epinephrine and AI-3 (Hughes et al. 2009). QseC directs expression of genes involved in cell metabolism, virulence, motility and stress responses (Hughes et al. 2009). To mediate these responses, QseC phosphorylates three distinct RRs, QseB, QseF, and KdpE (Hughes et al. 2009). QseB activates genes encoding flagella and motility (Sperandio et al. 2002a, b); QseF coordinates expression of genes encoding AE lesions and stress responses (Reading et al. 2007); and KdpE regulates genes encoding potassium uptake, osmolarity, and AE lesion formation (Nakashima et al. 1992; Hughes et al. 2009; Njoroge et al. 2012). Adrenergic signaling is essential for EHEC virulence during infection, as a *qseC* mutant strain is attenuated for virulence in rabbit-infection models (Clarke et al. 2006; Rasko et al. 2008).

9.8 The QseEF TCS

A second TCS involved in adrenergic signaling was identified in a microarray study that compared differential gene expression in WT and the *luxS* mutant EHEC strains. This TCS was renamed QseEF, where QseE is the HK and QseF is the RR (Reading et al. 2007). The *qseE* and *qseF* genes are encoded within a polycistronic operon that also contains the *yfhG* gene, which encodes an uncharacterized protein, as well as *glnB*, which encodes the PII protein involved in nitrogen regulation (Reading et al. 2007). QseE senses epinephrine and norepinephrine, as well as the environmental signals phosphate and sulfate, but does not sense AI-3. Therefore, QseE functions to sense strictly host-derived signals, in contrast to QseC that senses host- and bacterial-derived molecules (Clarke et al. 2006; Reading et al. 2009). Finally, QseC activates transcription of *qseEF*, and therefore, in the epinephrine and norepinephrine signaling cascade, QseE is downstream of QseC (Reading et al. 2007).

QseEF regulates expression of genes involved in the SOS response and Shiga toxin production, as well as transcription of genes encoding for other TCSs, including RcsBC and PhoPQ (Reading et al. 2009, 2010; Njoroge and Sperandio 2012). Additionally, QseEF influences AE lesion formation through regulation of EspFu/TccP (Reading et al. 2007). EspFu/TccP is an effector encoded outside of the LEE that enhances AE lesion formation (Campellone et al. 2004; Garmendia et al. 2004). The LEE-encoded T3SS translocates EspFu into the host cell where it mimics the eukaryotic SH2/SH3 adapter protein and leads to actin polymerization during AE lesion formation (Campellone et al. 2004). Bioinformatic analyses revealed that QseF contains a σ^{54} activator domain, whereas the *espFu* gene contains a conserved σ^{70} promoter, suggesting that QseF regulation of EspFu was indirect. Moreover, purified QseF did not bind to the espFu promoter in electrophoretic mobility shift assays. Together, these data confirmed that QseEF regula-

tion of EspFu requires an intermediate factor. Subsequent studies revealed that QseF regulates the sRNA GlmY, which is located immediately upstream from the *qseGFglnB* operon (Reichenbach et al. 2009). More recently, Gruber and Sperandio reported that GlmY, acting in concert with a second sRNA GlmZ, is the link between QseF and EspFu (Gruber and Sperandio 2014). Interestingly, GlmY and GlmZ promote *espFu* translation through cleavage of the transcript and negatively regulate expression of the *LEE4* and *LEE5* operons through destabilization of the mRNA (Gruber and Sperandio 2014).

9.9 Interplay Between QseBC and QseEF Sensing Systems

Single deletion strains of *qseC* or *qseE* are able to modulate gene expression in an epinephrine-dependent manner, whereas, *qseC/qseE* double mutant does not respond to epinephrine (Njoroge and Sperandio 2012). These findings suggest that QseC and QseE are the only adrenergic receptors in EHEC. QseC and QseE display convergent regulation of some target genes while differentially regulating others (Njoroge and Sperandio 2012). For example, QseB also promotes expression of GlmY, and thus regulates EspFu expression (Gruber and Sperandio 2014). Moreover, QseBC and QseEF negatively regulate expression of the TCS FusKR (Pacheco and Sperandio 2012). The HK FusK senses fucose in the GI tract, which EHEC uses to determine its location in the GI tract and correctly time expression of the LEE genes (Pacheco and Sperandio 2012). Further characterization of these regulatory cascades will provide a clearer understanding of how EHEC coordinates expression of these TCSs in order to precisely regulate virulence genes.

9.10 The Transcriptional Regulator QseA

QseA is a LysR-family transcriptional regulator that is activated by the AI-3/epi/NE signaling cascade (Sperandio et al. 1999, 2002a, b). QseA plays an important role in promoting ECHE virulence. QseA activates transcription of *ler*, and hence all the LEE genes (Sperandio et al. 2002a, b). The *LEE1* operon contains two promoters, a distal P1 promoter, and a proximal P2 promoter (Mellies et al. 1999; Sperandio et al. 2002a, b). QseA binds both promoters to regulate *ler* expression (Sperandio et al. 2002a, b; Kendall et al. 2010). Consistent with the transcriptional data, a *qseA* mutant strain formed significantly less AE lesions compared to WT EHEC (Sperandio et al. 2002a, b). Subsequent studies demonstrated that QseA regulates *grlRA* transcription in a Ler-dependent and Ler-independent mechanism and also showed that QseA regulon extends beyond the LEE and includes genes encoded in O-islands, which are regions of the chromosome unique to EHEC (Hayashi et al. 2001; Perna et al. 2001), other transcriptional regulators, sRNAs, as well as *qseE* (Reading et al. 2007; Kendall et al. 2010).

9.11 Disruption of AI-3/Epi/NE Signaling as an Antivirulence Strategy

Bacterial infections may lead to severe morbidity and mortality; however, the ability to treat these diseases with conventional antibiotics is becoming more and more limited. This is due primarily to the fact that antibiotics have lost their effectiveness as many bacteria are becoming resistant, often to multiple types of antibiotics. Conventional antibiotics disrupt essential functions, including DNA replication and protein synthesis, and thus place selective pressure on bacteria to develop resistance. An alternative approach may be to develop anti-virulence drugs that target bacterial virulence, but that does not inhibit bacterial growth or lead to death of the bacterial cell (Rasko and Sperandio 2010).

QseC homologues are present in over 25 plant and animal pathogens (Rasko et al. 2008). Thus, disrupting QseC signaling may be an effective strategy to inhibit virulence. Indeed, a high throughput screen identified a small, synthetic compound called LED209 (Fig. 9.1c) that blocked QseC signaling and prevented virulence expression not only in EHEC, but also in enteroaggregative *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Francisella tularensis* (Rasko et al. 2008; Curtis et al. 2014). LED209 functions as a prodrug that inhibits virulence by binding to and allosterically modifying QseC to disrupt activity (Curtis et al. 2014). LED209 specifically targets QseC and does not inhibit pathogen growth, suggesting that LED209 will not place selective pressure on pathogens to evolve resistance. An issue with an inhibitor of adrenergic signaling is that it may present adverse effects on the host. Significantly, LED209 did not present toxicity in cell culture or in rodents (Curtis et al. 2014), and future studies will need to be performed to confirm non-toxicity and efficacy in humans. Finally, some bacterial infections, including infections caused by *Clostridium difficile* and *Salmonella*, are associated with antibiotic use that disrupts the resident microbiota. Therefore, another important issue to be addressed concerns the effects of LED209 on the resident GI microbiota (Curtis and Sperandio 2011). Nevertheless, these recent findings underscore the potential of disrupting chemical signaling as a novel and effective antivirulence approach to treat diverse infectious diseases.

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Chapter 10
Mechanisms of Stress-Mediated Modulation of Upper and Lower Respiratory Tract Infections

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Abstract Stress is an external factor known to be a potent exacerbator of respiratory infections. Most explanations of how stress affects susceptibility to airway infections focus on the immune system. However, evidence is increasing that respiratory pathogens are equally responsive to the hormonal output of stress. This chapter considers the bacterial and mucosal determinants of respiratory tract infections and their interrelationship during stressful conditions.

10.1 Respiratory Immunity

Immune defenses of the upper respiratory tract encompass both host and bacterial factors (Wilson et al. 1996). Common to all mucosal surfaces, the symbiosis of resident bacteria and epithelial cell layer with its mucous layer protects the healthy steady state of the tissue. Studies using mice reared in a pathogen-free environment reveal that on introduction of bacteria, those that will become commensals are instrumental in shaping the mucosal immune repertoire (Ichimiya et al. 1991). Below the mucus layer that entraps particles and bacteria, there is a so-called periciliary layer composed of epithelial surface-bound mucins and glycolipids the state of hydration of which is a factor in optimal clearance function of the mucus, thereby preventing biofilm growth and subsequent inflammation (Randell et al. 2006). The periciliary fluid layer contains a multitude of antimicrobial factors (Grubor et al. 2006). Commensals are able to adhere to the mucus, thereby occupying a niche where they essentially discourage the claims of pathogens to the same environment they are said to compete. Mucociliary clearance by ciliary beat of specialized epithelial cells is especially important in the upper airway tract where

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Table 10.1 Innervation of the respiratory tree and its physiologic effects

Designation of nerves	Bronchial smooth muscle	Mucociliary system	Bronchial vessels
Parasympathetic nerves[1]	Constriction	Secretion	Dilation
Sympathetic nerves[2]	[a]		
Inhibitory nonadrenergic noncholinergic nerves[3]	Dilation		
Excitatory nonadrenergic noncholinergic nerves[4]	Constriction	Secretion Cough	Dilation Increase in permeability

The tone of the respiratory tree is under control of dominant, cholinergic-parasympathetic, adrenergic sympathetic mechanisms and non-adrenergic, non-cholinergic neural pathways (Joos, 2001). The effects are listed.

[a]No innervation, but expression of beta-adrenergic receptors, which trigger dilation on stimulation. The mediators are acetylcholine (1), norepinephrine (2), vasoactive intestinal peptide and nitric oxide (3), substance P and neurokinin A (4).

these cells are found – the lower respiratory tract (beyond the bronchial bifurcation) is essentially sterile and forms a significant innate immune barrier.

Respiratory epithelial cells are actively involved in the recognition of pathogens by their expression of pattern recognition receptors such as Toll-like receptors and secretion of antimicrobial peptides (Bals and Hiemstra 2004). Furthermore, epithelial cells are active players in instructing the respiratory immune response by physically anchoring intraepithelial dendritic cells, as well as synthesizing factors that influence T-cell mediated effector functions (Schleimer et al. 2007). Once the mucociliary escalator is impaired, and transit time for bacteria is prolonged, adherence to epithelial cells ensues and a potential respiratory infection begins. Production of virulence factors by the adherent bacteria leads to an extensive local respiratory immune response that affects intactness of mucociliary clearance, epithelial cell layer, IgA and function of attracted leukocytes (Wilson et al. 1996).

Nasal associated lymphatic tissue is the mucosal inductive site for humoral immune response of the upper respiratory tract (Zuercher et al. 2002). IgA or IgG deficiency, complement C4 deficiencies, common variable immune deficiency, and atopy all predispose the development of respiratory disease. It is widely known that virally-caused epithelial cell damage in itself can lead to bacterial super-infection by exposing neoligands for pathogen adherence.

10.2 Respiratory Mucosal Immunity, Neuronal Innervation, and Its Stress-Related Perturbations

The innervation of the respiratory tree is summarized in Table 10.1. Nervous system effectors play major roles in regulating respiratory function. For instance, airway muscle tone generally, and physiological reflexes relevant to infection

control such as coughing, bronchodilation, and mucociliary clearance, are all regulated by the sympathetic (adrenergic) and parasympathetic (cholinergic) branches of the autonomic nervous system (Nadel and Barnes 1984). Human tracheal gland cells respond to exogenous epinephrine and norepinephrine (Merten et al. 1993). Nebulized epinephrine is also used as a bronchodilator in intubated ventilated patients. Therefore, catecholamines will be present on host respiratory surfaces and so be components of the host secretions that will be detected by respiratory pathogens.

The connection between psychological status and airway health has long been recognized, and over the centuries realization of the need to reduce stress has incorporated in the treatment regime of both infectious and inflammatory respiratory disease, though usually without understanding the underlying biological mechanisms. For instance, in the pre-antibiotic era sanatoria practice, among others, environmental approaches to controlling tuberculosis (TB) involved low stress and emphasis on rest and relaxation techniques. Now, TB is less of a problem to the developed world due to usage of generally effective antibiotics. But, stress is still widely recognized as a predisposing factor to exacerbation of the disease, and re-activation of latent TB infections. In the modern context, asthma is a much more common human respiratory condition, and asthma sufferers worldwide are offered a multitude of supportive treatment methods beyond drugs only, aimed at influencing positively the psychological–endocrinological–immunological feedback loops now viewed as being important in the etiology of the disease.

Is the stress that can exacerbate TB or other respiratory conditions such as asthma causal or reactive in its effects? It is now widely believed that stress-related physiological reactions are contributory to the pathogenesis of a large number of clinical conditions. Importantly, stress may lead not only to a down-regulation of the humoral and cellular immune responses, but depending on the duration of stressor it may also impact the inflammatory threshold (Kemeny and Schedlowski 2007). Perception of stress in human normal and asthmatic subjects was found to positively correlate with the percentage of TNF-alpha producing T cells in the peripheral blood of the asthmatics, but not the control subjects (Joachim et al. 2007). Levels of TNF-alpha were also found to be elevated in chronic obstructive pulmonary disease (Barnes 2008), another condition affected by stress. It is important to understand that an increase in pro-inflammatory cytokines, when localized, is associated with tissue damage, which in itself increases the susceptibility to further injury (which can allergic, infectious, or fibrotic in origin). Unresolved, chronic inflammation predisposes respiratory disease patients to the development of the immunological phenomenon of epitope spreading, in which the adaptive immune response is widened, but paradoxically, remains specific. Such allergen cross-reactivity is thought to play a key role in the overall deterioration of health in patients suffering from asthma (Burastero 2006).

The physiological feedback loops, which are heightened in situations of stress, are the hypothalamus–pituitary–adrenal cortical axis and the sympathetic-adrenal-medullary axis (Chen and Miller 2007). These turn into pathophysiological feedback loops when cognitive and emotional evaluations of a perceived external

threat lead to an enhancement of inflammatory response. Cells of the immune system express receptors for catecholamine and glucocorticoid stress hormones, and other factors released during stress, such as neuropeptides (Reiche et al. 2004). Catecholamines and glucocorticoids have been shown to alter secretions or receptor densities of immune cells (Malarkey and Mills 2007). Airway macrophages have an activation profile that differs from other macrophages in the body, and possess receptors for epinephrine to which they respond in a way that increases severity of conditions such as asthma (James and Nijkamp 2000). Neuropeptides can also directly mediate stress-related inflammatory responses because they stimulate mast cells in local proximity to sensory nerve endings (Black 2002). Together, stress released hormones and peptides determine not only the duration of infectious disease, but also the sensitization of the respiratory tract, thereby changing the phenotype of initial airway disease. Stress hormones can also directly exacerbate inflammatory respiratory conditions. When mammals are exposed to repeat stress, alveolar macrophages can become pre-disposed to the development of a more inflammatory phenotype (Broug-Holub et al. 1998). This changes the respiratory milieu, making the organism more likely to show hyperreactivity to stimuli, which can in turn exacerbate the response to respiratory pathogens. Substance P is an example of a neuropeptide the release of which by bronchopulmonary nerves is increased on stress and in addition, is produced by inflammatory cells, which are increased in bronchoalveolar lavage in situations of stress (Joachim et al. 2006).

The lower respiratory tract in several respects represents an immunological entity in its own right because resident immune cells, in particular alveolar macrophages acting as antigen presenting cells and interacting T cells can both influence the type of immune response that favors antibody production, a so-called Th2 immune response. Furthermore, draining lymph nodes do not have a role in dealing with antigen, unlike other parts of the body, excepting immune privileged central nervous tissue. Rather, bronchus-associated lymphoid tissue, so-called BALT and, in the case of more chronic inflammation, tertiary lymphoid organs, assume a more predominant role in the immune response (Constant et al. 2002). Alveolar macrophages also contribute to the epinephrine content of broncho-alveolar lavage (Flierl et al. 2007) and are able to increase the production of epinephrine on activation.

Since stress is such a potent modulator of the immune response and its effector mechanisms (Elenkov et al. 2000), it follows that it may be a contributor to the success of immunization, a mainstay strategy in the prevention of respiratory infections such as influenza and pneumococcal disease. In a recent trial involving young adults receiving the hepatitis B vaccine, those subjects experiencing psychological distress had significantly lower specific antibody responsiveness (Marsland et al. 2006). Drummond and Hewson-Bower (1997) found a correlation between lower serum IgA/albumin ratio and stress in children with recurrent upper respiratory tract infections. Another study concluded that a healthy individual's increased sociability decreased the risk of viral upper respiratory tract infection (Cohen et al. 2003).

Relatively little is currently known about the impact of immune stressors on transmissibility and infectivity of bacteria colonizing the human respiratory mucosa. A recent murine study demonstrated that repeated stress led to prolonged, induced pathogen carriage, effecting delayed lethality on the first isogenic challenge but impaired protective immunity on second isogenic challenge (Gonzales et al. 2008). The implications of this chain of events for stress in the human work force may be considerable.

10.3 Stress and Its Influence on Susceptibility to Respiratory Infection via Modulation of Respiratory Pathogen Growth and Virulence

While the respiratory tract is usually seen to be in a "low state of alert" vis-à-vis the plethora of aerogenic stimuli (Holt et al. 2008), responding to an infectious organism brings with it the need to do this in a measured and balanced way, in order that the host may regain its steady state integrity, minimizing so-called bystander damage. This is a dual edged sword that the host is wielding and is exemplified by molecules like nitric oxide, which, on the one hand, is part of the respiratory tract's arsenal to respond to infectious organisms, which on the other hand, under excessive production and conversion to reactive metabolites can lead to tissue damage of the host (van der Vliet et al. 2000). Any stress-related tipping of this fine balance can lead to potentially deleterious local over-inflammation.

At the mucosal interface between host and microorganism, adhesion of bacteria to respiratory epithelial cells is influenced by very different stress-associated factors: bacterial adhesion is improved through expression of proteins that are induced by the paucity of iron which is effected by the host as a means of protection against iron-requiring pathogens (Perez Vidakovics et al. 2007). To the possible detriment of the host, bacterial binding to respiratory epithelia also tends to occur with greater efficiency in the hypersecretory conditions of inflamed airways, when the decoration of mucin glycoconjugates is altered (Davril et al. 1999).

Those microorganisms able to form biofilms are more likely to possess the ability to translocate the respiratory epithelial cell layer into deeper tissues than those that have lesser cohesive and adhesive organization (Yamazakki et al. 2006), and they thereby mediate the progression from inflammation to infection. It is therefore of interest that several of the immune effectors released during stress, particularly the catecholamines norepinephrine, epinephrine and dopamine, can induce changes in the bacterial phenotype relevant to biofilm formation such as adhesion to host epithelia (Vlisidou et al. 2004). In terms of the respiratory tract, catecholamines are naturally present in secretions due the role they play in regulating respiratory function (Table 10.1).

All patient groups share an increased risk of respiratory infection while in hospital; those in intensive care units receiving ventilation are at particular risk of developing so-called ventilation-associated pneumonia (Fridkin 1997). It is known that intubation/long dwelling catheters are associated with biofilm formation and

sepsis from bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Fridkin 1997). Interestingly, such bacteria have been shown to be receptive to the stress hormones that are elevated in acutely ill patients (Freestone et al. 2008a). What is also of significance is that patients in intensive care are frequently prescribed catecholamine inotropes, drugs that have been shown to increase commensal bacteria biofilm production in intravascular catheters (Lyte et al. 2003), as well as resuscitation of antibiotic-damaged pathogenic bacteria (Freestone et al. 2008b).

How might the catecholamines that are released into the respiratory mucosa during acute stress, or applied therapeutically, influence the infectivity of any potentially pathogenic bacteria within the vicinity of the elaborated hormone? The first context is growth. All pathogenic bacteria require iron to grow in vivo. This growth is prevented due to the presence of lactoferrin and transferrin, key innate immune defense proteins the role of which is to bind all free ferric iron, thereby making blood and respiratory secretions too low in iron to support the growth of iron-requiring pathogens. To overcome this usually very effective iron-limitation defense of host fluids, bacteria produce siderophores, very high affinity secreted ferric iron binding molecules, which allow them to acquire the transferrin and lactoferrin-sequestered iron. Work from several groups has shown that catecholamine stress hormones can also function as direct bacterial siderophores (Freestone et al. 2000, 2002) (see also Chap. 3). This stress hormone provision of iron can induce up to a million fold increase in the growth of pathogenic bacteria, including those causing airway infections such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and *Bordetella* species (Freestone et al. 1999; Alverdy et al. 2000; Anderson and Armstrong 2006, 2008). Epinephrine, norepinephrine, dopamine, and their metabolites can all reduce the iron-limiting function of bacteriostatic, key innate defense, proteins lactoferrin, and transferrin (Freestone et al. 2002). Through interaction of these host iron-binding proteins with stress hormones, blood and epithelial secretions are rendered much less bacteriostatic as bacteria are able to access normally inaccessible host sequestered iron sources (Alverdy et al. 2000; Freestone et al. 2000, 2002, 2008a; Anderson and Armstrong 2006, 2008). *Bordetella* species, significant respiratory pathogens of infants (causing whooping cough), also make use of stress hormones to increase transcription of several iron acquisition components (Anderson and Armstrong 2006, 2008). Dopamine, norepinephrine, and epinephrine are all able to induce the transcription of *bfeA*, the gene for the *Bordetella* enterobactin/catechol xenosiderophore receptor. In addition, as has been shown for many enteric and skin bacteria (Freestone et al. 1999), norepinephrine also stimulate growth of *Bordetella bronchiseptica* growth in iron-limiting medium containing serum, via enabling the bacteria to access transferrin bound iron (Anderson and Armstrong 2006, 2008). As already noted, respiratory secretions are markedly iron limited, though the action of host iron binding proteins, and catecholamines may therefore represent a route by which respiratory pathogens, such as *B. bronchiseptica,* could obtain essential iron in the host environment (Anderson and Armstrong 2006, 2008). Exposure of respiratory pathogens to stress-released hormones can also induce the bacteria to synthesize their own growth factors, the mechanism of action of which is non-transferrin dependent (Freestone et al. 1999). Thus, the effects of stress hormone exposure on bacteria can be manifest long after the initial stress event has ended, and catecholamine levels returned to normal.

10.4 Conclusion

Stress can influence susceptibility to respiratory infection through both the modulation of airway immune responses as well as direct effects upon the microbes that cause infections of the respiratory tract. Stress-mediated modulation of upper and lower respiratory tract disease is therefore multifactorial, encompassing host, microorganisms, and environmental influences. Although most of the microbial endocrinology research field has so far focused on bacteria resident in gut and skin tissues, it is now apparent that respiratory pathogens are also able to use stress hormone as direct environmental cues (Lyte et al. 2003; Anderson and Armstrong 2006, 2008; Freestone et al. 2008a). Because of the relative newness of the microbial endocrinology respiratory research field, it is unclear at present how the presence of stress hormones might shape the signaling events between host and bacterial species, and as a consequence the overall impact on mucosal immunity. However, it is clear that microbial endocrinology has the potential to lead to a better understanding of how emotional states can modulate susceptibility to both upper and lower respiratory tract infections.

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Chapter 11
Psychological Stress, Immunity, and the Effects on Indigenous Microflora

Michael T. Bailey

Abstract Psychological stress is an intrinsic part of life that affects all organs of the body through direct nervous system innervation and the release of neuroendocrine hormones. The field of PsychoNeuroImmunology (PNI) has clearly demonstrated that the physiological response to psychological stressors can dramatically impact the functioning of the immune system, thus identifying one way in which susceptibility to or severity of diseases are exacerbated during stressful periods. This chapter describes research at the interface between the fields of PNI and Microbial Endocrinology to demonstrate that natural barrier defenses, such as those provided by the commensal microflora, can be disrupted by exposure to psychological stressors. These stress effects are evident in the development of the intestinal microflora in animals born from stressful pregnancy conditions, and in older animals with fully developed microbial populations. Moreover, data are presented demonstrating that exposure to different types of stressors results in the translocation of microflora from cutaneous and mucosal surfaces into regional lymph nodes. When considered together, a scenario emerges in which psychological stressors induce a neuroendocrine response that has the potential to directly or indirectly affect commensal microflora populations, the integrity of barrier defenses, and the internalization of microbes. Finally, a hypothesis is put forth in which stressor-induced alterations of the microflora contribute to the observed stressor-induced increases in inflammatory markers in the absence of overt infection.

11.1 Introduction

It is well known that bidirectional communication exists between the brain and the peripheral organs such that the central nervous system (CNS) can impact organ functioning, and physiological changes in the body can affect the CNS.

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However, the extent of this communication, the mechanisms through which they occur, and the impact on health are still only beginning to be defined. Current research within the field of PsychoNeuroImmunology (PNI) has clearly shown that different emotional states, or exposure to psychological stressors, are associated with enhanced susceptibility or increased severity of diseases through nervous system-induced alterations in innate and adaptive immunity. And, it is becoming evident that other more primitive defenses, such as the intestinal microflora, are also affected by exposure to psychological stressors (Freestone et al. 2008). Moreover, stressor-induced bacterial translocation of microflora from mucosal surfaces to secondary lymphoid organs may lead to inflammation and/or altered activation of adaptive immunity. This chapter describes the effects of psychological stressors on the gastrointestinal (GI) tract and presents data showing that the stress response affects the number of bacteria residing as part of the intestinal microflora and their ability to translocate to regional lymph nodes. These findings will be discussed within the context of host defense against infectious diseases.

11.2 Psychological Stress, the Stress Response, and the Impact on Immunity

Stress is an intrinsic part of life, and successfully adapting to stimuli that induce stress is necessary for the survival of an organism in its environment that is constantly changing. Although there is not a commonly used definition of stress, the concept of stress is often broken down into the challenge (called the stressor) and the behavioral and physiological responses to this challenge (called the stress response). A stressor is any stimulus that disrupts internal homeostasis, and can involve psychological, physical, or physiological stimuli. Initiation of the response to physiological and physical stressors is often subconscious and completely biological in nature. But, psychological stressors evoke an additional cognitive processing where the stressors must first be encoded as exceeding the organism's ability to cope with the demand. This cognitive processing sets into motion a coordinated behavioral and physiological response that is similar to the response to physiological and physical stressors. Two neuroendocrine pathways are major contributors to the stress response, namely, the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS). Activation of the HPA results in increased circulatory levels of adrenocorticotrophic hormone (ACTH) produced by the pituitary gland as well as mineralcorticoid and glucocorticoid hormones derived from the adrenal cortex. In contrast, SNS activation results in the release of norepinephrine (NE) from sympathetic nerve termini in SNS innervated tissues, including the GI tract and lymphoid tissues. As such, periods of stress are associated with increases in circulating glucocorticoid hormones (primarily cortisol in humans and corticosterone in rodents) as well as increased circulating and tissue levels of NE. These hormones have a variety of effects throughout the body, such as mobilizing energy for the well known "fight-or-flight" response, that are all aimed at helping the body respond to the demands being placed on it.

Research in the field of PNI has amply demonstrated that stressful periods are associated with exacerbations of a variety of different diseases. For example, it has been demonstrated that individuals reporting higher levels of stress in their daily lives are more likely to develop clinical symptoms during experimental respiratory viral infection (Cohen 2005). To determine if these effects are due to stressor-induced immunosuppression, many researchers have studied the immune response to vaccination during stressful situations, and have found that stressors influence antibody and T-cell responses to vaccines. For example, it was demonstrated in medical students that responsiveness to hepatitis B vaccination was significantly reduced during final exams, an effect found to be associated with stress perception and feelings of loneliness (Glaser et al. 1992; Jabaaij et al. 1996). Likewise, the chronic stress associated with caring for a spouse with Alzheimer's disease (AD) resulted in lower antibody responses to influenza vaccination (Kiecolt-Glaser et al. 1996). Determining the mechanisms through which these stressors affect immune reactivity in humans is difficult, but many animal studies demonstrate that stressor-induced hormones are in fact responsible for the stressor-induced exacerbations of infectious diseases. For example, stressor-induced elevations in corticosterone have been found to suppress lymphocyte trafficking and cytokine production during influenza viral infection (Dobbs et al. 1996; Hermann et al. 1995), as well as antigen processing and presentation by dendritic cells infected with recombinant vaccinia virus (Elftman et al. 2007; Truckenmiller et al. 2005, 2006). The anti-inflammatory effects of glucocorticoid hormones are now well known, and it is evident that glucocorticoid hormones suppress inflammatory cytokine production in part through negative regulation of NF-κB activation and function (Sternberg 2006).

The catecholamines can also have immunomodulatory effects through activation of adrenergic receptors. Animal models have demonstrated that adrenergic signaling is responsible for stressor-induced suppression of cytolytic CD8+ T cell responses during influenza viral infection (Dobbs et al. 1993). Likewise, an acute cold/restraint stressor significantly suppressed the CD4+ T cell response to *Listeria monocytogenes* infection through a β1-adrenergic receptor mediated mechanism (Cao et al. 2003). Ex vivo and in vitro data has revealed that catecholamine stimulation of β-adrenergic receptors at the time of immune challenge, suppresses cytokine production, NK cell activity, and T cell proliferation. In this case, cAMP is thought to be involved in this catecholamine induced immunosuppression (Padgett and Glaser 2003).

Under some circumstances, though, stressors can also enhance certain components of the immune response, particularly the innate immune response. For example, Lyte et al. (1990) demonstrated that exposing mice to a social stressor, called Social Conflict, significantly increased the phagocytic capacity of elicited peritoneal macrophages (Lyte et al. 1990). And, rats exposed to acute shock as a stressor produce higher levels of nitric oxide upon subcutaneous bacterial challenge (Campisi et al. 2002). Because in vitro studies have shown that culturing macrophages with NE increases phagocytosis (Garcia et al. 2003) and the production of nitric oxide (Chi et al. 2003), it is likely that stressor-induced increases in phagocyte activity are NE dependent.

These studies reflect the complex nature of the impact of neuroendocrine hormones on the immune response. The field of microbial endocrinology (Lyte 2004) has added an additional layer of complexity by demonstrating that microbes themselves can be influenced by stressor-induced hormones. Moreover, research by our group and by others have shown that more primitive defense mechanisms, such as microbial barrier defenses at cutaneous and mucosal surfaces, can also be affected by the stress response. These studies are a logical extension of previous findings within the fields of PNI and microbial endocrinology, and will be discussed within the context of stress physiology and infectious disease.

11.3 Overview of the Indigenous Microflora

The human body harbors an enormous microflora that even in the healthy host grossly outnumbers cells of the body by a factor of 10 (i.e., approximately 10^{14} bacterial cells:10^{13} human cells) (Berg 1996, 1999). These bacteria are generally referred to as the microflora and colonize all external surfaces of the body, such as the skin, oral and nasal cavities, upper respiratory tract, urinary tract, and reproductive tract. The GI tract, however, is the main reservoir of bacteria and harbors roughly 90% of the microflora. Molecular analysis of the intestinal microflora using 16s ribosomal RNA have increased previous culture-based estimates of between 400–500 species in the intestines to as high as 1,800 genera and 15,000–36,000 different individual species (Frank et al. 2007). As a result of this high bacterial load and great diversity, the microflora genome is estimated to contain more than 100 times as many genes as the human genome (Gill et al. 2006).

The microflora of the body are not simply opportunistic colonizers or potential pathogens. Rather, the microflora are true symbiotic organisms that have many beneficial effects on the host. Although metabolic activities have been attributed to the intestinal microflora, such as the synthesis of vitamin K and vitamin B complex and the conversion of precarcinogens and carcinogens to noncarcinogens, many studies have focused on the importance of the intestinal microflora for maintenance of mucosal immunity. These effects have been well studied using germ free mice, which are known to have reduced levels of serum immunoglobulins, smaller Peyer's patches, fewer intraepithelial lymphocytes, and a diminished capacity to produce cytokines (reviewed in (Shanahan 2002)). Interestingly, introducing intestinal microflora to these germ free mice restores many (but not all) components of the mucosal immune system. (Stepankova et al. 1998; Gordon et al. 1997; Umesaki et al. 1993, 1995).

In addition to stimulating GI physiology and mucosal immunity, the intestinal microflora can directly prevent diseases by creating a barrier to potential pathogens. Colonization exclusion of new strains of bacteria from the external environment is an essential function of the microflora and disruption of this barrier can facilitate pathogen colonization. Two bacterial types are often associated with colonization exclusion, members of the genus *Bifidobacterium,* and members of the genus *Lactobacillus*.

Ely Metchnikoff speculated nearly 100 years ago that lactic acid bacteria (such as *Lactobacillus* spp.) were health-promoters, able to limit pathogen colonization and proliferation (Metchnikoff 1908). The development of reliable in vitro models has helped to define the mechanisms through which the microflora provide protection. And, it is now known that attachment of *Lactobacillus acidophilus*, *Bifidobacterium breve*, and *B. infantis* to intestinal cells creates a physical barrier to enteric pathogens, such as enteropathogenic *Escherichia coli*, *Yersinia pseudotuberculosis*, and *Salmonella typhimurium* (Bernet et al. 1993; Coconnier et al. 1993a, b). Moreover, ingestion of probiotic bacteria, i.e., bacteria ingested for their beneficial effects, such as probiotic lactobacilli, significantly affects the LD_{50} of many enteric pathogens (Coconnier et al. 1998; Bernet-Camard et al. 1997; Hudault et al. 1997) and reduces the severity of experimental infection with *Helicobacter pylori* or *Citrobacter rodentium* (Johnson-Henry et al. 2004, 2005). As such, our studies focused primarily on assessing the impact of psychological stress on the levels of *Lactobacillus* spp. and *Bifidobacterium* spp.

11.4 Stress-Induced Alterations in Intestinal Microflora

The number and types of bacteria that reside as part of the indigenous microflora are thought to be relatively stable, but environmental and physiological challenges have been shown to disrupt this stability. For example, early studies by Schaedler and Dubos (1962) demonstrated that rehousing mice into new cages significantly decreased lactobacilli levels (Schaedler and Dubos 1962). And, chronic sleep deprivation in rats was shown to induce a significant overgrowth of microflora in the ileum and cecum (Everson and Toth 2000), with more recent studies indicating that intrinsic factors such as age and gender can also affect the composition of the microflora of laboratory animals (Ge et al. 2006). Fewer studies have focused on environmental affects on the microflora of humans, but an early study in cosmonauts demonstrated that the intestinal microflora were significantly affected during space flight (Lizko 1987), with others suggesting that some of the effects could be due to the stress of confinement (Holdeman et al. 1976). To further study the potential impact of psychological stress on the stability of the intestinal microflora, we assessed the microflora of young rhesus monkeys that were being separated from their mothers for husbandry purposes (Bailey and Coe 1999).

In captive colonies, rhesus monkeys are routinely separated from their mothers at approximately 6 months of age. At this age, the monkeys are no longer nursing and are eating solid foods. Yet, they still show a strong physiological and emotional reaction to separation from their mothers. This transition from living with the mother to living with other peer monkeys is associated with an increased incidence of diseases, including GI diseases. While much of this can be explained by exposure to new contagion or the actions of the nervous system on the immune system, we hypothesized that the stress response during maternal separation could significantly affect microflora levels in the infants, and thus reduce the barrier effects of the intestinal microflora.

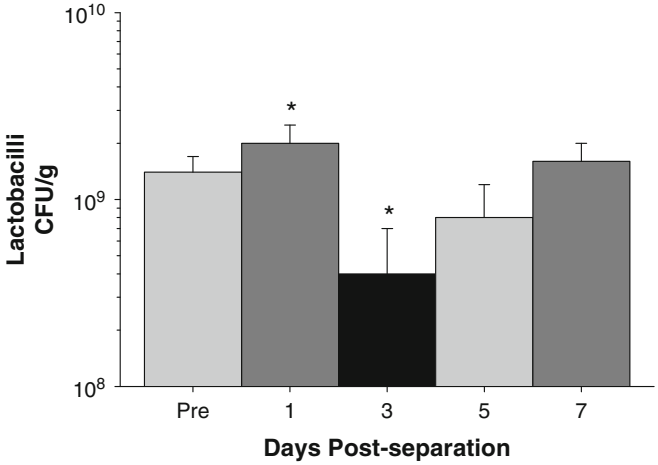

Fig. 11.1 Aerobically grown lactobacilli were enumerated from coprocultures before and for 1 week following maternal separation. Results are mean (S.E.) number of colony forming units (CFU) per gram of fecal matter (wet weight). *$p < 0.05$ versus preseparation values. Reproduced from Developmental Psychobiology, 1999 with permission from Wiley

Culture-based enumeration of shed microflora revealed significant alterations in bacterial levels the week following maternal separation compared to levels when the infants were still residing with their mothers. This was evident for Gram-negative and total aerobic and facultatively anaerobic microflora, but only reached statistical significance when a single genus of bacteria was enumerated. The number of aerobically grown lactobacilli was significantly altered after maternal separation (Fig. 11.1). In most cases, the alterations followed a standard profile of increased levels immediately after separation, followed by significantly lower levels 3 days after separation and a return to baseline by the end of the week. Interestingly, the magnitude of the reduction in microflora 3 days after maternal separation could be predicted by the infants' behavior on day 2 post-separation. Three stress-indicative behaviors, cooing, barking, and lip smacking, were associated with microflora levels; in general, those animals that had the highest number of stress-indicative behaviors shed the fewest lactobacilli and total aerobic and facultatively anaerobic bacteria on day 3 post-separation (Fig. 11.2) (Bailey and Coe 1999).

Lactic acid bacteria, such as members of the genus *Lactobacillus*, are thought to be important contributors to microflora-mediated colonization exclusion. Thus, stressor-induced reductions in lactobacilli would be hypothesized to be associated with enhanced susceptibility to enteric infection. In this experiment, none of the monkeys were intentionally infected, but many nonhuman primate colonies have endemic levels of enteric pathogens, notably *Shigella flexneri* and *Campylobacter jejuni*. And, 45% (i.e., 9/20) of the infant monkeys became colonized with either *S. flexneri* or *C. jejuni* during the week following maternal separation. On the first day

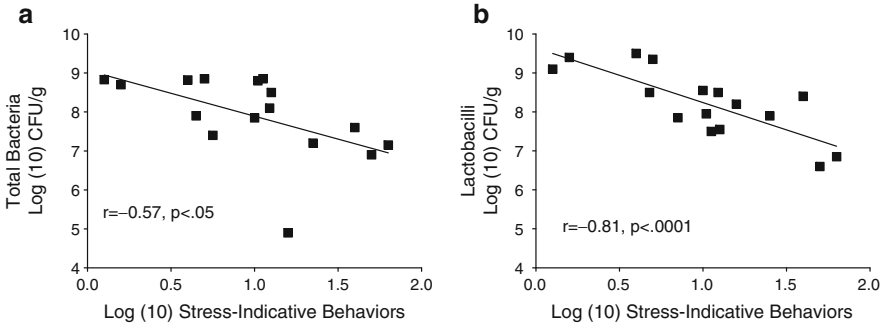

Fig. 11.2 Log transformed stress-indicative behaviors were significantly associated with log(10) CFU/g of intestinal microflora. (**a**) Total aerobic and facultatively anaerobic microflora. (**b**) Aerobically grown lactobacilli. Reproduced from Developmental Psychobiology, 1999 with permission from Wiley

that pathogen colonization was observed there was a weak, marginally significant ($p=0.07$) inverse association between the number of lactobacilli and pathogens shed from the intestines (Bailey and Coe 1999). These data are consistent with the idea that lactobacilli are important in colonization resistance against enteric pathogens, but further studies are needed to conclude that stressor-induced alterations in microflora result in increased susceptibility to enteric infection.

Stressor-induced reductions in lactobacilli have also been found in college students during stressful periods (Knowles et al. 2008). In this study, lactobacilli levels were determined during a low stress period (i.e., the first week of the semester) and a high stress period (i.e., final exam week). The exam period was associated with significantly higher levels of perceived daily stress and weekly stress, as well as an increase in GI upset. Moreover, when compared to the low stress period, levels of lactic acid bacteria, primarily lactobacilli, shed in the stool were significantly lower for up to 5 days following examination, with differences in bacterial levels reaching one half log unit in magnitude (e.g., baseline values of 6×10^7 CFU/ml vs. 1×10^7 on day 5 post-examination). It should be noted, however, that significant differences in diet did occur across the two time periods; most notable were significant reductions in vegetable consumption and a significant increase in coffee consumption (Knowles et al. 2008). But, given that stressor exposure alters lactobacilli levels in laboratory animals fed a standardized diet, it is likely that stress-associated changes in human microflora reflect an impact of the stressor as well as potential effects of diet.

Healthy adults are somewhat resistant to the impact of stressors on various physiological systems. For example, stressor-induced alterations in the immune response tend to return to baseline upon termination of the stress response. However, the stress response can have a more prolonged effect on immunity in the very old and the very young (Coe and Lubach 2003). And, stressor exposure in the very young, or even during the prenatal period, is thought to set the infant on a

significantly different developmental trajectory, resulting in larger stressor induced effects later in life (Coe and Lubach 2003). One of the most consistent findings in regards to exposure to prenatal stressors is that fetal growth and birth weight are reduced after women experience stressful situations during pregnancy (Field et al. 1985; Lederman et al. 1981; Lederman 1986). Rhesus monkeys have been used extensively to investigate the influence of prenatal stress on infant development. And, it has been shown that prenatal stress affects nueromotor development (Schneider and Coe 1993), emotional reactivity to stressors (Clarke and Schneider 1993), brain monoamine levels (Schneider et al. 1998), cell density in the brain (Coe et al. 2002, 2003), and immune reactivity (Coe et al. 1996, 1999, 2007). Our studies focused on the impact of gestational stress on the intestinal microflora across the four phases of microflora development.

Bacteria colonize the GI tract of newborns in a sequential pattern that is tightly related to developmental milestones in the infant (Cooperstock and Zed 1983). The first phase of colonization begins at birth when bacteria from the mother's reproductive tract colonize the otherwise sterile newborn. These bacteria do not predominate for long and are quickly overcome by maternal aerobic intestinal microflora, which are thought to persist in the intestines for the first few days of life (Tannock et al. 1990). These aerobic species, such as *E. coli* and *Streptococcus* spp. consume molecular oxygen as they grow and begin to reduce the oxidation–reduction potential in the intestines creating a more favorable environment for the growth of anaerobic species (Meynell 1963). As a result, high levels of Enterobacteriaceae are evident 1 day after birth, but anaerobes, such as bifidobacteria, predominate by 6 days of age and throughout the period of exclusive breast feeding (Sakata et al. 1985).

Members of the genus *Bifidobacterium* thrive in breastfed infants and are the predominant bacteria in the intestines due to growth factors found in human milk that bifidobacteria readily use for energy, such as lactose. As bifidobacteria grow, they produce pronounced levels of lactic and acetic acids that can not be buffered by human milk, thus inhibiting the growth of acid sensitive microbes. Breast milk also contains large amounts of immune factors, such as secretory immunoglobulins, lactoferrin, lysozymes, and even leukocytes that can inhibit colonization of certain bacteria (Balmer and Wharton 1991; Wharton et al. 1994a, b). The combination of immune factors and acidic fermentation products gives bifidobacteria a tremendous ecological advantage over other species (Heine et al. 1992; Beerens et al. 1980).

The initiation of weaning from breast milk is associated with a resurgence of aerobic and facultatively anaerobic species, such as *E. coli*, *Streptococci*, and *Clostridia* spp., that are naturally found in newly ingested foods. The concentrations of these newly arrived bacteria fluctuate greatly during this period, but as the diet becomes more consistent, microbial populations in the intestines also stabilize and will remain quite stable throughout the lifespan. This stability is important for maintaining intestinal homeostasis (O'Hara and Shanahan 2006), and if disrupted could contribute to the development of GI infections or cancers (O'Hara and Shanahan 2006).

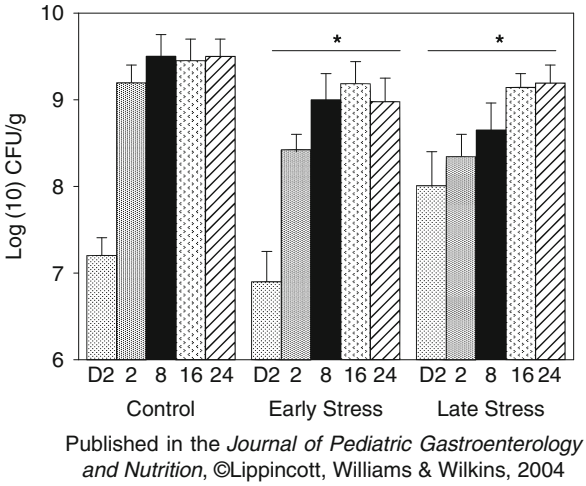

Fig. 11.3 Anaerobically grown *Lactobacillus* spp. during the first 24 weeks of life. Data are the mean (SE) of log(10) transformed number of colony forming units per gram of fecal matter (CFU/g). Concentrations on day 2 of life were not significantly different between pregnancy conditions. * Both Early Stress and Late Stress infants had significantly fewer anaerobic lactobacilli than did control infants across the first 24 weeks of life ($p<0.05$). In addition, there was a developmental trend for increasing titers across the 24 week period in both control and prenatally stressed infants ($p<0.05$). Reproduced from Journal of Pediatric Gastroenterology and Nutrition, 2004, with permission from Lipincott, Williams, & Wilkins

11.5 Prenatal Stressor-Induced Alterations to Microflora Development

To determine the impact of a prenatal stressor on microflora development, an acoustical startle stressor (i.e., 3 random 110 dB beeps over a 10 min period occurring 5 days per week) was used to evoke a stress response from pregnant rhesus monkeys either early (days 50–92) or late (days 105–147) in the 169 day gestational period. These periods represent crucial time periods in nervous system and GI system development, thus making it likely that disruption of physiological homeostasis at these time points affects fetal development. This stressor resulted in a significant increase in cortisol in the pregnant mothers, but did not appear to significantly affect the number of miscarriages, gestational length, or birth weight (Bailey et al. 2004b). The stressor did, however, significantly affect the development of the intestinal microflora.

During the first 6 months of life, lactobacilli levels in the monkeys born from mothers exposed to the stressor during gestation were significantly lower than levels found in infants from non-stressed control mothers, with the biggest differences in mean levels found at 2 weeks of age (Fig. 11.3). As successful nursing progressed, bifidobacteria began to predominate in the intestines. And, as with the lactobacilli, bifidobacteria levels were significantly lower in the intestines

of infant monkeys from mothers that were exposed to the acoustical startle stressor during gestation. This effect, however, was only evident in the offspring from mothers exposed to the stressor late in gestation (Fig. 11.3) (Bailey et al. 2004b). As with the previous study involving maternal separation, none of the monkeys in this study were intentionally infected with enteric pathogens. However, approximately 43% of the infants from mothers stressed early in gestation and 12% of infants from mothers stressed late in gestation became subclinically colonized with *Shigella flexneri*, an endemic pathogen in the monkey colony. Importantly, Shigella were not detected in any of the infants born from the non-stressed control condition (Bailey et al. 2004b), suggesting that prenatal stress, particularly late in gestation, disrupted the development of natural resistance to the enteric pathogen, *S. flexneri*.

11.6 Psychological Stress and the GI Tract: Toward a Mechanism of Stressor-Induced Alterations in Microflora

It is tempting to speculate on the mechanisms through which the intestinal microflora could have been altered by stressful pregnancy conditions. For example, it is known that cortisol can affect many aspects of infant development, and many of the effects of prenatal stress on the immune system can be mimicked by administration of ACTH or the synthetic glucocorticoid, dexamethasone (Coe et al. 1996). And, others have found that giving corticosterone to pregnant rats significantly reduced the concentrations of total and Gram-negative aerobes and facultative anaerobes (Schiffrin et al. 1993). The mechanisms through which glucocorticoids might affect the microflora are not known, but fetal development of the gi tract is thought to be influenced by glucocorticoids. For example, maturation of the intestines occurs concomitantly with the prepartum surge in cortisol in precocial species, such as pigs, sheep, and humans (Trahair and Sangild 1997). Moreover, very high levels of glucocorticoids adversely affect intestinal development, such as the ability to secrete gastric acid and the densitivity of villi and crypts (Sangild et al. 1994), thus changing the microenvironment in the intestines and opening the possibility of shifts in ecological competition.

Altering the microenvironment may also affect established microflora populations in older hosts. The complete set of factors controlling the types of bacteria that can reside as part of the intestinal microflora are not well understood, but it is thought that the host plays a role in "selecting" the microflora. This was elegantly demonstrated by Rawls et al. (2006), who reciprocally transplanted gut bacteria between mice and zebrafish. After transplantation, gut microbial populations shifted to reflect the proportions of bacteria found in the microflora of conventionally reared recipient animals, and no longer reflected the microflora of the original

donor animal (Rawls et al. 2006). This may in part be due to the physiology of the host GI tract, since certain aspects of GI physiology are known to influence microbial populations in the GI tract. For example, it is well known that for bacteria to take up residence in the gi tract, they must first survive the low pH of the stomach. Therefore, it is not surprising that reduced production of gastric acid (as occurs with hypochlorhydria) results in overgrowth of bacteria in the gi tract (Drasar et al. 1969). Some species, however, such as members of the genera *Lactobacillus* and *Bifidobacteria* are acid tolerant and are able to grow in the low pH (Drasar et al. 1969). This acid tolerance gives the genera an ecological advantage over other species that compete to colonize the gi tract. Therefore, a logical hypothesis is that any stimulus that disrupts gastric acid production will in turn affect intestinal microflora levels.

The influences of emotional states on the secretion and motility of the GI system were documented as early as 1833, when the surgeon William Beaumont noted that the secretion of gastric juice was decreased or abolished during periods of anger or fear in his patient with a gastric fistula (Beaumont 1838). Experimental data has confirmed this observation, and it is now known that secretion of gastric acid can be suppressed by experimental stressors, such as the cold pressure task and mental arithmetic (Badgley et al. 1969; Holtmann et al. 1990). In animals, different stressors have differential effects on acid secretion, with restraint stress reported to significantly increase or decrease gastric acidity depending upon temperature (Murakami et al. 1985; Lenz et al. 1988). These differences are due to different levels of activation of the sympathetic and parasympathetic nervous systems; activating the SNS suppressed whereas activating the PNS enhanced acid secretion (Yang et al. 2000). Research is needed to determine whether gi acidity plays a role in stressor-induced alterations of microflora.

There are, of course, additional secretory products that can affect microflora levels and are themselves influenced by the stress response such as additional digestive products like bile, and immune products like secretory immunoglobulin A (sIgA) and antimicrobial peptides. The use of secretory immunoglobulin deficient mice has shown the importance of this immunoglobulin in influencing microbial populations; sIgA deficient mice have significantly increased populations of anaerobic microflora in the small intestine (Fagarasan et al. 2002). Moreover, antimicrobial peptides, such as the defensins, have been suggested to modify the types and numbers of bacteria colonizing the GI tract (Salzman et al. 2007). Because these molecules can be affected upon exposure to a stressor (Jarillo-Luna et al. 2007; Korneva et al. 1997), an additional plausible hypothesis is that stress-associated alterations of the microflora are dependent upon stressor-induced alterations in sIgA and/or defensins.

Perhaps the most well-studied effects of stress on the gi tract are the effects on GI motility. Animal models have established that stress reduces gastric emptying (Taché et al. 2001; Nakade et al. 2005) and slows transit in the small intestine (Lenz et al. 1988; Kellow et al. 1992) through stressor-induced elevations of corticotrophin releasing hormone (Taché et al. 2001; Nakade et al. 2005). In contrast to the

inhibitory effects in the stomach and small intestine, stress tends to enhance motility in the colon due to increased sacral parasympathetic outflow to the large intestine through a CRH dependent circuit (Lenz et al. 1988; Martinez et al. 1997).

Gastrointestinal motility has long been thought to influence microbial populations in the GI tract. For example, slowing peristalsis, and thus motility, by administering high doses of morphine causes significant bacterial overgrowth in the small intestines of rats (MacFarlane et al. 2000; Scott and Cahall 1982). Moreover, data from humans show an association between surgical trauma, stagnation of intestinal motility, and bacterial overgrowth, thus supporting the notion that delayed intestinal motility can result in bacterial overgrowth (Marshall et al. 1988; Nieuwenhuijzen et al. 1996a, b). Interestingly, increased GI motility can also affect microflora levels, with some studies showing a direct correlation between small intestine microflora levels and the rate of peristalsis.

An equally likely explanation is that the intestinal microflora were directly affected by stressor-induced increases in intestinal hormones, such as NE. The primary focus of this book is the exciting finding that bacteria can change their growth characteristics when exposed to hormones. And, the growth of many types of microflora has been shown to be significantly enhanced upon culture with NE (Freestone et al. 2002). Despite the many studies showing bacterial growth enhancement by NE in vitro, demonstrating that these interactions occur in vivo has been challenging. Neuroendocrine–bacterial interactions, however, undoubtedly occur in vivo when NE levels reach high levels. This was evident with the use of the neurotoxin 6-hydroxydopamine, which lyses the nerve terminals of sympathetic neurons resulting in the release of NE that is stored in the nerve terminals (Lyte and Bailey 1997). Thus, even though 6-OHDA is a useful way to chemically sympathectomize laboratory rodents, its initial effect is the release of a large bolus of NE 24 h after injection (Porlier et al. 1977; De Champlain 1971). Interestingly, bacterial levels in the cecums of mice were found to be significantly increased 24 h after administration of 6-OHDA, with *E. coli* showing the greatest increase (Lyte and Bailey 1997). Since the growth of commensal *E. coli* is strongly affected by exposure to NE (Freestone et al. 2002), the data suggest that overgrowth of *E. coli* in the cecums of chemically sympathectomized mice results from direct enhancement of bacterial growth by NE.

Overgrowth of bacteria in the family Enterobacteriaceae is also evident in the intestines of mice exposed to psychological stressors. Our recent studies indicate that restraining mice for prolonged periods (i.e., 16 h per day for 7 days) result in an overgrowth of Enterobacteriaceae in both the small and large intestines (Bailey et al. manuscript under review) as well as in the cecum (Bailey et al. 2006). This overgrowth may have important health implications, since bacterial overgrowth is a precipitating factor in the translocation of bacteria from the gi tract to the rest of body. In fact, the translocation of some species in the family Enterobacteriaceae was found to be directly related to levels in the small intestine and cecum (Steffen and Berg 1983). The finding that exposing mice to psychological stressors can enhance *E. coli* levels in the intestines prompted the determination of the impact of psychological stressors on bacterial transloction.

11.7 Stressor-Induced Bacterial Translocation

Indigenous microflora are not invasive bacteria, which is one property that allows them to reside with their host. Moreover, the external surfaces of the body, i.e., cutaneous and mucosal surfaces, maintain a barrier to external substances, including microbes. In mucosal tissues, transport of solutes into the body is controlled in part through tight junctions between intestinal epithelial cells that prevent the passive transfer of molecules and microbes. Bacteria from mucosal surfaces, however, are routinely sampled by specialized phagocytic cells, called M cells, which engulf mucosal bacteria and pass them to regional lymph nodes in order to initiate an immune response or to maintain tolerance. Most of these bacteria are killed en route, resulting in low to undetectable levels of culturable bacteria in regional lymph nodes.

The epidermal layer of the skin is also well known to provide a permeability barrier that primarily serves to prevent water loss in a potentially desiccating environment. This barrier, however, is also a potent barrier to the passage of cutaneous microflora into the body. As a result, normal mice rarely have detectable levels of bacteria in lymph nodes that drain cutaneous surfaces. In our studies, less than 15% of non-stressed control mice were found to have bacteria in the inguinal lymph nodes that lie under skin in the lower back (Bailey et al. 2006). This percentage was significantly increased when mice were exposed to prolonged restraint or the social stressor, social disruption (SDR), with 82% of mice in both groups identified as having bacteria in these draining lymph nodes. To try to determine whether this effect could simply be due to mechanical breaches in the skin (such as from biting during SDR, or abrasions from the restraint tube), a separate group of mice received full thickness skin biopsies on the lower back. Interestingly, only 36% of mice in this group were found to have bacteria in the inguinal lymph nodes, which was significantly less than the 82% occurrence in the stressed animals (Bailey et al. 2006). These data indicate that the stress response, rather than mechanical barrier breaches, is responsible for the bacterial translocation of cutaneous microflora.

The percentage of mice with bacteria cultured from mesenteric lymph nodes, which drains the GI tract, was higher than the percentage of mice found to have bacteria in the inguinal lymph nodes. We found that 48% of non-stressed control mice were culture-positive for bacteria in the mesenteric lymph nodes. However, exposure to either restraint or SDR increased the occurrence of bacteria in the mesenteric lymph nodes to over 80% (i.e., 82% of SDR mice were culture positive; 91% of restrained mice were culture positive). Interestingly, depriving the mice of food and water did not significantly affect the translocation of indigenous microflora, indicating that the stress response, rather than other physiological variables significantly enhanced bacterial translocation in the gut (Bailey et al. 2006).

There are now several reports indicating that barrier defenses in both the skin and the GI tract can be disrupted by exposure to psychological stressors. Acute experimental stressors in human participants, such as a public speaking tasks and sleep deprivation, were shown to disrupt the permeability barrier in the skin as determined by measuring transepidermal water loss (TEWL) and by determining the water

content of the outermost layer of the skin, i.e., the stratum corneum (Altemus et al. 2001). In mice, TEWL was also found to be affected by exposure to different housing conditions and by immobilization stress (Denda et al. 2000). This effect was later found to be due to the impact of glucocorticoid hormones on the stratum corneum (Choi et al. 2006). In addition to physical barrier properties, the skin also produces many antimicrobial peptides, such as β-defensins and cathelicidins. And, these antimicrobial peptides have been shown to be suppressed during stressor exposure through the actions of stressor-induced glucocorticoids and local production of corticotrophin releasing hormone (Aberg et al. 2007). Thus, stressor-induced alterations in the skin permeability barrier, as well as innate defenses, may explain why bacteria were found in the inguinal lymph nodes of stressed mice.

Exposure to psychological stressors can have similar effects in the GI tract, with stressor-induced changes in gut permeability being well defined (Soderholm and Perdue 2001). These effects have primarily been described in laboratory animals, since studying the impact of psychological stressors on GI permeability in humans has been challenging. Several studies have demonstrated that immobilizing rats in a cold environment significantly increased jejunal permeability to ^{51}Cr-EDTA and mannitol (Saunders et al. 1994) via a cholinergic dependent mechanism (Saunders et al. 1997; Soderholm and Perdue 2001). In the colon, permeability was also increased by immobilization in a cold environment, an effect that could be mimicked by peripheral injection of CRH (Saunders et al. 2002; Soderholm and Perdue 2001).

Enhanced microflora growth and increased permeability of barrier defenses may not be sufficient to result in bacterial translocation. In fact, an additional important component of bacterial translocation is the ability of enteric bacteria to adhere to intestinal tissue. Interestingly, stressor-induced neuroendocrine hormones can also enhance the attachment of enteric bacteria. For example, culturing pathogenic *E. coli* O157:H7 with NE significantly increased the ability of the bacteria to adhere to colonic tissue (Chen et al. 2003; Green et al. 2004). Moreover, internalization of pathogenic (i.e., *Salmonella choleraesuis* and *E. coli O157:H7*), but not necessarily commensal, bacteria was enhanced by treating porcine Peyer's patch mucosa with NE in an Ussing chamber paradigm (Green et al. 2003).

These studies demonstrate that exposure to psychological stressors affects many aspects of host physiology. And, many of these effects have the capacity to alter the commensal microflora. When considered together, a likely scenario emerges from the data in which exposure to a psychological stressor results in a neuroendocrine response that has the potential to directly or indirectly affect commensal microflora populations, the integrity of barrier defenses, and the internalization of microbes. Delineating whether the effects of stress on the microflora are direct effects, i.e., whether stress hormones themselves affect microflora in vivo, or indirect, i.e., through modulation of the microenvironment in which commensals interact with their host, will be a challenge for future studies. However, as animal models to study the interactions between the microflora and their host continue to be developed, insight into the impact of stress on these interactions will undoubtedly follow.

11.8 An Integrative Hypothesis of Stress, Infection, and Immunity

The importance of stressor-induced alterations in commensal microbial populations and translocation to regional lymph nodes is only beginning to be understood. Although the ability of microbial populations to limit pathogen colonization and invasion has been known for many years, it is now thought that these commensal microbes help to regulate the immune system as well. In GI tissue, for example, inflammation is low despite the enormous antigenic potential of the billions of colonized bacteria. These commensal bacteria, though, may actually be active players in maintaining homeostasis through the suppression of innate pattern recognition receptor signaling. For example, signaling through the Toll-like receptors (TLR), which results in cytokine production, is negatively regulated through Toll-interacting protein (Tollip) and single immunoglobulin IL-1R-related molecule (SIGGR) (O'Hara and Shanahan 2006). Importantly, Tollip expression is directly correlated with microflora levels; the highest levels of Tollip are found in healthy colonic tissue that also has the highest microflora levels (O'Hara and Shanahan 2006; Otte et al. 2004). Thus, as long as healthy levels of microflora are maintained within the GI lumen, inflammation may be actively suppressed.

If the stress response facilitates the passage of bacteria from the lumen into the intestinal tissues, however, the TLRs found on the basolateral surface of enterocytes would then be able to respond to translocating bacteria to initiate an inflammatory response (Abreu et al. 2001; Otte et al. 2004). Moreover, there is an immense network of phagocytes and antigen presenting cells residing below the enterocytes within the GI tissue that can respond to translocating microbes and cause a local or systemic inflammatory response. Thus, it is possible that innate receptors, such as the TLRs, are silenced in the presence of normal luminal levels of microflora, but activated when microflora levels are altered or when they translocate into the tissue.

The ability of stressors to induce and/or enhance the inflammatory response is now well recognized. For example, stressor-induced elevations in circulating inflammatory markers have been found in uninfected humans (Brydon et al. 2005, 2006; Steptoe et al. 2007; Coussons-Read et al. 2007; Bierhaus et al. 2003) as well as in uninfected rodents (Avitsur et al. 2001, 2002; Bailey et al. 2007; Engler et al. 2008; Stark et al. 2001). In rodents, it has been shown that exposure to certain stressors causes leukocytes, especially monocytes/macrophages, to become resistant to the suppressive effects of corticosterone (Avitsur et al. 2001; Bailey et al. 2004a; Engler et al. 2005; Stark et al. 2001). Moreover, these cells have a primed phenotype and show exaggerated inflammatory responses to ex vivo stimulation with LPS or even intact bacteria (Avitsur et al. 2003; Bailey et al. 2007), effects that were associated with the inability of glucocorticoids to suppress the activation of the transcription factor NF-κB (Quan et al. 2003). Human studies employing gene chip technology corroborate findings from murine studies, and have found an underrepresentation of genes containing a glucocorticoid response element and an

overrepresentation of genes controlled by the transcription factor NF-κB (which controls the transcriptional expression of inflammatory cytokines) in uninfected individuals reporting high levels of psychological stress (Miller et al. 2008; Cole et al. 2007). These data suggest that periods of psychological stress in humans are associated with an inflammatory profile that is not able to be controlled by endogenous glucocorticoids.

The question remains, however, why cells of the innate immune system become activated in the absence of an active infection. While it has been shown that treating cells in culture with NE can result in the production of cytokines (Tan et al. 2007), most immunologists would argue that activation of cells with inflammatory stimuli, such as microbes or microbe-associated molecules, is necessary for the production of appreciable amounts of cytokines. Given the impact of stressors on microbial populations and translocation of microbes or microbe-associated molecules, like LPS, into the body, a reasonable hypothesis is that stressor-induced alterations and translocation of the indigenous microflora activates and/or primes the immune system and are partly responsible for stressor-induced elevations in circulating inflammatory cytokines. One remaining challenge for the field of microbial endocrinology is to test this hypothesis and determine whether stressor-induced alterations of commensal microflora can shift the balance from health to disease.

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Chapter 12
The Epinephrine/Norepinephrine/Autoinducer-3 Interkingdom Signaling System in *Escherichia coli* O157:H7

Cristiano G. Moreira and Vanessa Sperandio

Abstract Epinephrine/norepinephrine/AI-3 signaling is used as an interkingdom chemical signaling system between microbes and their hosts. This system is also exploited by pathogens to regulate virulence traits. In enterohemorrhagic *E. coli* (EHEC) O157:H7, it is essential for pathogenesis and flagella motility. These three signals activate expression of a pathogenicity island named locus of enterocyte effacement (LEE), Shiga toxin, and the flagella regulon. These signals are sensed by the two-component system QseBC, whereas the bacterial membrane receptor QseC autophosphorylates and phosphorylates the QseB response regulator initiating a complex phosphorelay signaling cascade that activates the expression of a second two-component system, QseEF. The QseEF two-component system is also involved in the expression of the virulence genes, and it senses epinephrine, phosphate, and sulfate. This complex signaling cascade still needs to be completely elucidated.

12.1 *Escherichia coli* O157:H7

E. coli is one of the most well-studied bacterium in the microbiology field due to its frequent incidence in different environments and hosts, as well as its use as a tool in molecular biology. Currently, there are several categories of *E. coli* known to cause disease, mainly diarrhea in humans, also named as diarrheiogenic *E. coli*. Among those, enterohemorrhagic *E. coli* O157:H7 (EHEC) is one of the most important pathogenic *E. coli*. EHEC has been associated with several recent food-borne outbreaks of bloody diarrhea and hemolytic uremic syndrome (HUS) throughout the world. EHEC has an unusually low infectious dose when compared with other enteric bacterial pathogens such as *Vibrio cholera* and *Salmonella*

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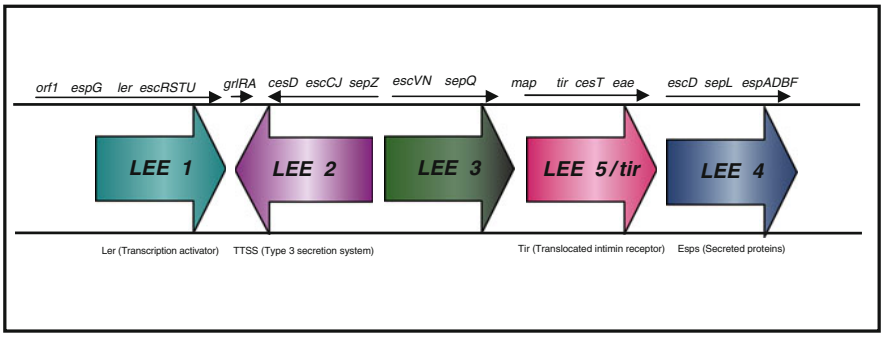

Fig. 12.1 (**a**) The locus of enterocyte effacement (LEE) pathogenicity Island found in EHEC, which encodes factors responsible for type III secretion and pedestal formation. *LEE1* encodes for *ler*, the LEE-encoded regulator. *LEE1, LEE2*, and *LEE3* encode for factors involved in type III secretion. *LEE4* encodes for EspA, EspB, and EspD. The *LEE5/tir* operon encodes for intimin and Tir (McDaniel et al. 1995; Kenny et al. 1997a; Mellies et al. 1999)

enterica Typhimurium. EHEC colonizes the large intestine and produces a potent toxin, Shiga toxin (Stx), responsible for the hemorrhagic colitis and HUS, which can culminate in kidney failure and leads to the mortality associated with EHEC outbreaks (Kaper et al. 2004).

EHEC causes a histopathological lesion on intestinal epithelial cells called attaching and effacing (AE). The AE lesion is characterized by the destruction of the microvilli and the rearrangement of the cytoskeleton to form a unique pedestal structure that cups the bacterium individually (Fig. 12.1a).

Chemical signaling through the AI-3/Epinephrine/Norepineprhine signals activates expression of virulence genes in EHEC. Most of these virulence genes are involved in the formation of the AE lesion and are contained within a pathogenicity island named the locus of enterocyte effacement (LEE) (McDaniel et al. 1995) (Fig. 12.2).

The EHEC LEE region contains 41 genes, most of which are organized into five major operons: *LEE1, LEE2, LEE3, LEE5*, and *LEE4* (Elliott et al. 1998; Elliott et al. 1999; Mellies et al. 1999). The LEE encodes a type III secretion system (TTSS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990), and this adhesin's receptor, the translocated intimin receptor (Tir) (Kenny et al. 1997b), which is translocated into the epithelial cell through the bacterial TTSS (Elliott et al. 1998; Elliott et al. 1999; Mellies et al. 1999) (Fig. 12.1a).

The TTSS is an apparatus that spans the inner and outer bacterial membranes forming a microscopic "needle." Several proteins, including EscD, EscR, EscU, EscV, EscS, and EscT span the inner membrane and associate with a cytoplasmic ATPase, EscN, which is required for secretion of proteins (Roe et al. 2003). EscC is predicted to form the main protein ring in the outer membrane to which the EscF "needle" is connected (Wilson et al. 2001). EscF comprises the syringe connected to the filament of the translocon. The translocon consists of EspA, which creates a sheath around the EscF needle. EspB and EspD are located at the distal end of the

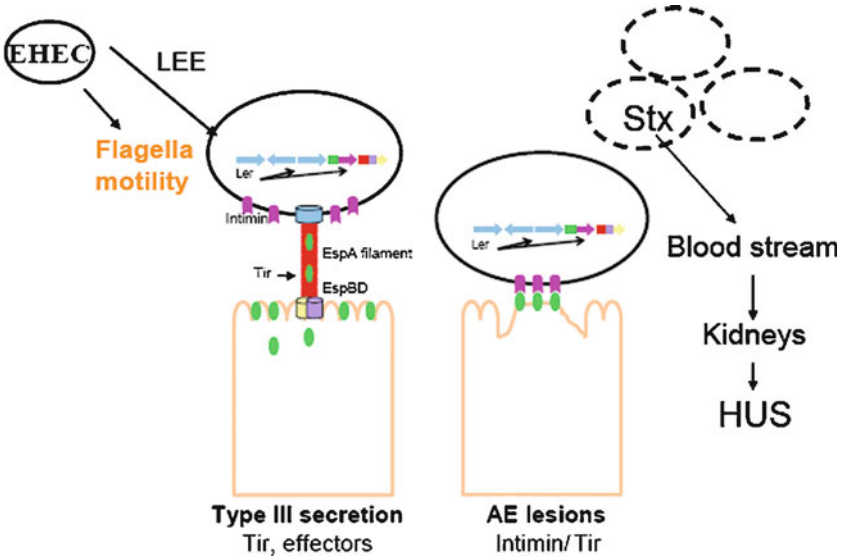

Fig. 12.2 General model for EHEC pathogenesis. LEE region of EHEC encodes most of T3SS effectors, which are essential for EHEC pathogenesis, as well as flagella motility. Ler is master regulator of LEE, intimin is an outer membrane protein, through T3SS EHEC translocates Tir, Intimin-Tir binding culminates in histopathologic lesion called attaching and effacing lesion (or lesion AE). Shiga toxin in EHEC plays late role during infection that can cause hemolictic uremic sindrome (Kaper et al. 2004)

TTSS and form 3–5 nm pores in the host cell membrane (Ide et al. 2001) through which translocated proteins are secreted.

The *eae* gene (*E. coli* attaching and effacing) encodes for intimin, an outer membrane protein that acts as an intestinal adherence factor (Jerse et al. 1990). Mutants of the *eae* gene are defective in intimate adherence to intestinal epithelial cells, which prevents the concentration of polymerized actin necessary for the development of AE lesions. The translocated intimin receptor (Tir), which is also encoded in the LEE, is translocated from the bacterium through the TTSS into the host cell to serve as a receptor for intimin (DeVinney et al. 1999; Kenny and Finlay 1995; Rosenshine et al. 1996). In the host cell membrane, Tir adopts a hairpin loop conformation and serves as a receptor for the bacterial surface adhesin, intimin (Deibel et al. 1998). Binding of intimin to Tir promotes the clustering of N- and C-terminal cytoplasmic regions and leads to the initiation of localized actin assembly beneath the plasma membrane (Campellone et al. 2004). The EHEC Tir recruits the host protein N-WASP (Goosney et al. 2001) through an interaction with EspFu, another bacterial protein encoded within a prophage, which is also translocated through the TTSS into the host cell (Campellone et al. 2004).

The TTSS encoded by the LEE translocates LEE-encoded and non-LEE encoded effectors. The mitochondrial associated protein, *map*, affects the integrity of the host mitochondrial membrane (Kenny and Jepson 2000) and is encoded

directly upstream of *tir*. Another effector, EspF, is responsible for the disruption of the intestinal barrier function and induces cell death by an unknown mechanism (McNamara and Donnenberg 1998; McNamara et al. 2001). EspG is responsible for the disruption of microtubule formation and plays a role in virulence in the rabbit enteropathogenic *E. coli* (REPEC) model (Tomson et al. 2005), while EspH, which is encoded in *LEE3*, is responsible for the modulation of the host cell cytoskeleton through the inhibition of cell cycle signals (Tu et al. 2003). Although encoded outside the LEE pathogenicity island, several effector proteins have been recently shown to be secreted through the EHEC TTSS (Tobe et al. 2006). These include Cif, which induces host cell cycle arrest and reorganization of host actin cytoskeleton (Charpentier and Oswald 2004), and NleA, which has been shown to localize to the Golgi and play a key role in virulence in an animal model (Gruenheid et al. 2004).

EHEC also produces a powerful Shiga toxin (Stx) that is responsible for the major symptoms of hemorrhagic colitis and HUS. The Stx family contains two subgroups, Stx1 and Stx2. Stx1 shows little sequence variation between strains (Zhang et al. 2002), whereas antigenic divergence has been observed among the Stx2s, including Stx2, Stx2c, Stx2d, and Stx2e (Perera et al. 1988; Schmitt et al. 1991; Zhang et al. 2002). Stx2 has been more associated epidemiologically with severe human disease than Stx1 (Boerlin et al. 1999), with Stx2 and Stx2c being most frequently found in patients with HUS (Ritter et al. 1997).

The genes encoding Stx1 and Stx2 are located within the late genes of a λ-like bacteriophage and are transcribed when the phage enters its lytic cycle (Neely and Friedman 1998). Once the phage replicates, Shiga toxin is produced, and the phage lyse the bacteria, thereby releasing the toxin into the host. The bacteriophage enters its lytic cycle during an SOS response triggered by disturbances in the bacterial membrane, DNA replication, or protein synthesis (Kimmitt et al. 1999; Kimmitt et al. 2000). These triggers are all common targets of conventional antibiotics and may contribute to the controversy surrounding the use of antibiotics to treat EHEC-mediated disease. Shiga toxins consist of a 1A:5B noncovalently associated subunit structure (Donohue-Rolfe et al. 1984). The B subunit of Stx is known to form a pentamer that binds to the eukaryotic glycolipid receptor, globotriaosylceramide (Jacewicz et al. 1986; Lindberg et al. 1987; Waddell et al. 1988). The A subunit is then cleaved by trypsin and reduced, resulting in a polypeptide that causes depurination of a residue in the 28S rRNA of 60S ribosomes (Endo et al. 1988). This leads to the inhibition of protein synthesis, injury of renal glomerular endothelial cells, and the initiation of a pathophysiological cascade that leads to HUS (Fig. 12.2).

12.2 Transcriptional Regulation of the LEE Region

Chemical signaling plays an important role in LEE and flagella expression (Sperandio et al. 1999; Sperandio et al. 2001). The bacterial signal, autoinducer-3 (AI-3), produced by the human intestinal microbial flora, the epinephrine and norepinephrine host stress hormones, as well as the flagella regulon activate expression

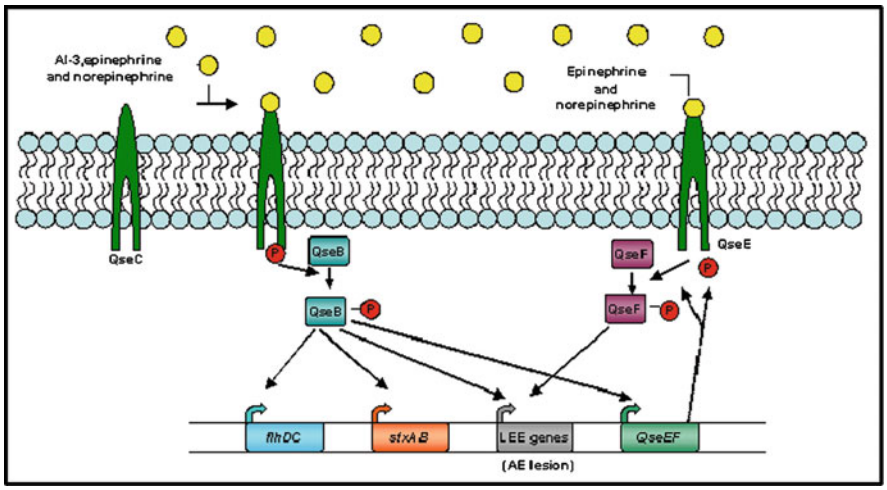

Fig. 12.3 Model for regulation of autoinducer (AI)-3, epinephrine, and norepinephrine (NE) bind the bacterial membrane receptor QseC, which results in its autophosphorylation. QseC then phosphorylates its response regulator QseB and initiates a complex phosphorelay signaling cascade that activates the expression of a second two-component system (QseEF), the locus of enterocyte effacement (LEE) genes, which encode various proteins, including the components of a type III section system that are involved in attaching and effacing (AE) lesion formation, the motility genes (flhDC), and Shiga toxin (stxAB). The QseEF two-component system is also involved in the expression of the LEE genes, and although its activators have not yet been elucidated, it is possible that it senses epinephrine and/or NE (Hughes and Sperandio 2008)

of the LEE genes (Sperandio et al. 2003). These signals act agonistically to increase LEE gene expression (Walters and Sperandio 2006) Fig. 12.3.

In addition to the regulation via stress hormones, other transcription factors are involved on LEE regulation. Iyoda and Watanabe (2004) have observed that EHEC encodes the genes *pchA, pchB,* and *pchC* (PerC homologs) that positively activate the expression of the LEE genes (Iyoda and Watanabe 2004). *LEE1* encodes for Ler, the LEE-encoded regulator, which was shown to be required for the expression of all genes within the LEE (Bustamante et al. 2001; Elliott et al. 2000; Kaper et al. 2004; Mellies et al. 1999). Another important factor in the regulation of the LEE is the integration-host factor, or IHF. IHF has been shown to be required for the expression of the entire LEE through the direct activation of *ler* transcription (Friedberg et al. 1999). Additionally, EtrA and EivF are two negative regulators of the LEE region, acting possibly through *ler* transcriptional repression. The *etrA* and *eivF* genes are found within a second pathogenicity island that encodes a cryptic type III secretion system (ETT2) (Zhang et al. 2004). The histone-like nucleoid-structuring protein, H-NS, is responsible for the repression of *LEE2, LEE3,* and *LEE5* transcription in the absence of Ler (Bustamante et al. 2001; Haack et al. 2003). RpoS, a stationary phase sigma factor, activates the transcription of the *LEE3* operon (Sperandio et al. 1999). Finally, Hha has been reported to repress the transcription of the *LEE4* operon (Sharma and Zuerner 2004).

Two previously uncharacterized genes in the LEE region, *orf10* and *orf11*, were recently renamed GrlR, global regulator of LEE repressor, and GrlA, global regulator of LEE activator (Barba et al. 2005; Deng et al. 2004). This study suggested that GrlA is responsible for the transcriptional activation of *ler*, while GrlR represses *ler*. Additionally, it is known that Ler activates the transcription of *grlRA* (Barba et al. 2005; Elliott et al. 2000) and that GrlRA activates the expression of *LEE2* and *LEE4*, independently of Ler (Russell et al. 2007).

12.3 Quorum Sensing in EHEC

Surette and Bassler reported in 1998 quorum sensing signaling in *E coli* K12 and *S. enterica* Typhimurium (Surette and Bassler 1998) through the production of autoinducer-2 (AI-2) by these bacteria (Surette et al. 1999). A common gene on these bacterial species was cloned and identified as responsible for AI-2 production, and it was named *luxS* (Surette et al. 1999). Later on, LuxS was characterized as the enzyme involved in the metabolism of *S*-adenosylmethionine and shown to convert ribose–homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione, which is the precursor of AI-2 (Schauder et al. 2001). Currently, the autoinducer referred to as AI-2 is a furanosylborate-diester in *Vibrio harveyi* (Chen et al. 2002), and a 2R, 4S-2-methyl-2,3,3,4-tetrahydrofuran (R-THMF) for *Salmonella* sp.(Miller et al. 2004).

However, at that time, no phenotypes have been shown to be regulated by quorum sensing in *E. coli* and *Salmonella* sp. In 1999, Sperandio et al. (1999) described that quorum sensing signaling activated transcription of the LEE genes and type three secretion in pathogenic *E. coli*, both in EHEC and in enteropathogenic *E. coli* (EPEC). It was then proposed that signaling through AI-2 might be involved in virulence gene regulation in *E. coli*. In 2001, quorum sensing was shown to be a global regulatory mechanism in EHEC, involved in AE lesion formation, motility, metabolism, growth, and Shiga toxin expression (Sperandio et al. 2001). The EHEC's quorum sensing regulatory cascade started to be unraveled with the description of the first three quorum sensing regulators (Sperandio et al. 2002a; Sperandio et al. 2002b), which were named Quorum sensing *E. coli,* Qse regulators. QseA was reported as a transcription factor from the widespread LysR family, directly involved in activation of *LEE1* (*ler*) transcription and EHEC's pathogenesis (Russell et al. 2007; Sharp and Sperandio 2007; Sperandio et al. 2002a). The QseBC regulatory system was described as a novel two-component system involved in quorum sensing regulation of flagella expression and motility (Clarke and Sperandio 2005b; Sperandio et al. 2002b).

A year later, it was shown that AI-2 was not the autoinducer involved in EHEC virulence regulation. The signal identified was a yet undescribed autoinducer, which was named autoinducer 3 (AI-3). In addition, it was reported that the eukaryotic hormones epinephrine and/or norepinephrine could substitute for the bacterial AI-3 signal to activate expression of the EHEC virulence genes. Among them, *ler*,

the master regulator of LEE, was shown to be activated in presence of AI-3. Moreover, flagella motility of EHEC regulated via QseBC system was demonstrated to be affected by AI-3. Given that eukaryotic cell-to-cell signaling occurs through hormones, like epinephrine and norepinephrine, this cross talk between bacteria and host seem to happen through cross-signaling between quorum sensing signals and host hormones (Sperandio et al. 2003).

12.4 Infectious Disease and Hormones

Since the 1920s, hormones have been used for treatment of a variety of illnesses (Yamashima 2003). Following studies have shown the role of stress hormones directly on bacterial functions.

Catecholamines, such as epinephrine and norepinephrine, are chemical compounds derived from the amino acid tyrosine containing catechol and amine groups. They are stress hormones involved in the fight or flight response. They are synthesized from L-dopa into dopamine, then norepinephrine, and epinephrine. Both norepinephrine and dopamine containing sympathetic nerve terminals are distributed throughout the body, including the intestinal tract, where they are part of the enteric nervous system. Epinephrine synthesis is restricted to the central nervous system and adrenal glands. However, epinephrine is released into the bloodstream, especially during stress, acting systemically in the whole body (Furness 2000).

Both epinephrine and norepinephrine are recognized by adrenergic receptors in mammalian cells. Adrenergic receptors are a subset of the G-protein coupled receptors (GPCRs) family, which are transmembrane receptors coupled to heterotrimeric guanine-binding proteins (G proteins). Adrenergic receptors are divided in two main subtypes: α and β. Both epinephrine and norepinephrine are recognized by a very similar ligand-binding pocket in all adrenergic receptors (Cherezov et al. 2007; Freddolino et al. 2004).

12.5 QseC: A Bacterial Functional Analog of an Adrenergic Receptor

Unlike mammalian cells, EHEC does not encode a GPCR receptor in its genome; hence, sensing of adrenergic hormones by this bacterium was occurring through another type of receptor. The main signaling transduction systems in bacterial cells are the two-component systems. In these systems, the sensor for environmental cues is a histidine kinase (usually membrane bound), which upon autophosphorylation transfers its phosphate to an aspartate residue in the response regulator. This response regulator is usually a transcription factor that is activated by phosphorylation. QseC is a histidine sensor kinase that augments its phosphorylation specifically in response to the AI-3 bacterial quorum sensing signal and the host

hormones epinephrine and/or norepinephrine (Clarke et al. 2006). Importantly, tritiated norepinephrine was shown to specifically bind to QseC, and this binding could be antagonized only by a λ-adrenergic antagonist (phentolamine), while a β-adrenergic antagonist (propranolol) did not have any effect on QseC activity (Clarke et al. 2006). QseC then transfers its phosphate to its cognate response regulator QseB, which only upon phosphorylation binds to its target promoters to regulate gene expression (Clarke et al. 2006; Clarke and Sperandio 2005a; b).

12.6 QseBC Two-Component System

The QseBC system was initially described as a two-component system regulated by quorum sensing, which shares homology with *Salmonella enterica* serovar Typhimurium PmrAB (Sperandio et al. 2002b). The same study showed that QseBC was involved in regulation of flagella and motility in EHEC.

In a detailed study (Clarke and Sperandio 2005a), it was shown that QseBC constituted a two-component system, and that the *qseBC* genes were cotranscribed forming an operon. Moreover, it was reported that QseB autoactivated transcription of the *qseBC* operon (Clarke and Sperandio 2005a). Using primer extension, the start site of the *qseBC* transcript was mapped, and through nested deletion analysis it was determined the minimal region necessary for QseB transcriptional autoactivation. Also, electrophoretic mobility shift assays, competition experiments, and DNAse I footprints showed that QseB directly binds to 2 sites in its own promoter.

Additionally, Clarke and Sperandio (2005b) described that QseBC regulates the flagella expression and motility through *flhDC*, the master regulator of flagella. Using electrophoretic mobility shift assays, competition experiments, and DNAse I footprints, it was shown that QseB directly binds to the *flhDC* promoter both in low and high affinity binding sites. In this study, it was also reported that the promoter of *flhDC* responsive to QseBC had a σ^{28} consensus. In summary, these studies suggested that transcription of the *flhDC* promoter by QseBC is a complex system and is dependent on the presence of FliA (σ^{28}).

QseC's homologs are found at least in 25 species of bacteria, including animals and plant pathogens (Table 12.1), suggesting that this signaling system is not restricted to *E. coli* (Rasko et al. 2008). A few recent reports have implicated these non-EHEC *qseC* homologues in virulence gene activation presumably through a combination of AI-3, norepinephrine, and epinephrine activation. The chemical structure of AI-3 was not completely elucidated yet. However, the role of epinephrine and norepinephrine has been extensively reported in different strains of *E. coli, Salmonella, Staphylococcus*, among others. A *qseC* homologue was shown to contribute to virulence in a mouse infection model of the class A bioterrorism threat agent *Francisella tularensis* (Weiss et al. 2007). A *qseC* homologue was also demonstrated to be involved in norepinephrine dependent enhancement of motility and colonization of juvenile pigs by *S. enterica* serovar *Typhimurium* (Bearson and Bearson, 2008) and virulence gene expression in this bacterium (Merighi et al. 2006; Merighi et al. 2009).

Table 12.1 Some of bacterial genera and species where QseBC (Rasko et al. 2008) and QseEF two-components systems are conserved, other than EHEC

QseBC	QseEF
Enteropathogenic *E. coli*	Uropathogenic *E. coli*
Enteroaggregative *E. coli*	*E. coli* k12
E. coli k12	*S. enterica* Typhimurium
Salmonella	*Salmonella enterica* Typhi
Vibrio	*Salmonella enterica* Paratyphi
Shigella	*Shigella flexneri*
Enterococcus	*Shigella boydii*
Campylobacter	*Shigella dysenteriae*
Yersinia	*Shigella sonnei*
Psychrobacter	*Bacillus subtilis*
Fransciella	*Bacillus amyloliquetaciens*
Erwinia carotovora	
Hemophilus influenzae	
Pasteurella multocida	
Actinobacillus pleuropneumoniae	
Chromobacterium violaceium	
Rubrivivax gelatinosus	
Thiobacillus denitrificans	
Ralstonia eutropa	
Ralstonia metallidurans	

12.7 The QseEF Two-Component System

Recently, a second two-component system in the AI-3/epinephrine/norepinephrine signaling cascade was described by Reading et al. (2007) and named QseEF. The *qseE* and *qseF* genes are part of a polycistronic operon that also contain in this cluster the *yfhG* gene, which encodes for uncharacterized protein, and *glnB*, which encodes the PII protein involved in nitrogen regulation (Reading et al. 2007). Transcription of *qseEF* is activated by epinephrine through QseC, suggesting that QseEF comes second to QseBC in the hierarchy of gene expression in this signaling cascade.

In this system, QseE is the sensor kinase, and QseF the response regulator. QseF activates transcription of the recently described gene encoding EspFu (Reading et al. 2007), an effector protein of EHEC, which is encoded outside the LEE region. EspFu is translocated into the host cell by EHEC where it mimics an eukaryotic SH2/SH3 adapter protein to engender actin polymerization during pedestal formation (Campellone et al. 2004). In silico analysis indicates that QseF contains a σ^{54} activator domain. However, the *espFu* gene contains a conserved extended σ^{70} consensus promoter region, suggesting that QseF indirectly activates transcription of *espFu*. In agreement to this hypothesis, eletrophoretic mobility shift experiments demonstrated that QseF does not bind to the *espFu* promoter region. These data showed that QseF-dependent activation of *espFu* transcription is indirect and involves an intermediary factor transcribed in a σ^{54}-dependent fashion.

Reading et al. (2007) then showed that the QseEF two-component system, involved in *espFu* transcriptional activation, is essential for AE lesion formation. This system is also found in other bacterial species. However, it is restricted to enteric bacteria not being as widespread as QseBC (Table 12.1).

12.8 Qse A Regulator

Sperandio et al. (1999) reported that the LEE genes from EHEC were activated by quorum sensing through Ler, which is encoded by the first gene of the *LEE1* operon (Sperandio et al. 1999). QseA belongs to the LysR family of transcription factors and shares homology with the AphB and PtxR regulators of *V. cholerae* and *Pseudomonas aeruginosa*, respectively (Sperandio et al. 2002a). This study reported that QseA activates transcription of *ler* and consequently all the LEE genes. The *LEE1* operon has two promoters, a distal promoter P1 (163 base pairs upstream of the translational start site), and a proximal promoter P2 (32 base pairs upstream of the translational start site). QseA acts on the distal (P1) promoter of *LEE1*. An EHEC *qseA* mutant also presented a remarkable reduction on the type three secretion system. Hence, QseA is part of the AI-3/epinephrine/norepinephrine regulatory cascade that regulates the EHEC LEE region via transcriptional activation of *ler*. Altogether, this study showed that QseA activates the transcription of *LEE1* by directly binding upstream of its P1 promoter region.

In 2007, Russell et al. (2007) reported yet another level of LEE regulation through QseA. They reported that *grlRA* transcription is activated by QseA in both a Ler-dependent as well as a Ler-independent fashion, adding another layer of complexity to the regulation of the LEE genes (Russell et al. 2007).

12.9 Future Implications of the AI-3/Epinephrine/ Norepinephrine InterKingdom Signaling in EHEC Pathogenesis and Development of Therapeutics

The growing worldwide challenge of antimicrobial resistance and the paucity of novel antibiotics underscore the urgent need for innovative therapeutics. The increasing understanding of bacterial pathogenesis and intercellular communication, when combined with contemporary drug discovery tools and technologies, provides a powerful platform for translating such basic science into therapeutic applications to combat bacterial infections. Interference with bacterial cell-to-cell signaling via the quorum-sensing pathway constitutes an especially compelling and novel strategy since it also obviates the development of bacterial resistance. Quorum sensing allows bacteria to respond to hormone-like molecules called autoinducers and is responsible for controlling a plethora of virulence genes in several bacterial pathogens. Because quorum sensing is not directly involved in essential

processes such as growth of the bacteria, inhibition of quorum sensing should not yield a selective pressure for development of resistance. Quorum sensing antagonists confuse or obfuscate signaling between bacteria and bacteria and host and, unlike antibiotics, do not kill or hinder bacterial growth. Hence, quorum sensing antagonists should be viewed as blockers of pathogenicity rather than as antimicrobials.

QseC is a receptor for the AI-3/epinephrine/norepinephrine signals and is central for the pathogenesis of EHEC. In addition, QseC homologs are present in many bacterial pathogens of animals and plants (Table 12.1). Finally, QseC is involved in quorum sensing and adrenergic signaling, and this signaling is not directly involved in processes essential for bacterial growth. Thus, in theory, inhibitors of QseC would not induce selective pressures promoting evolution of bacterial resistance.

Indeed a recent report demonstrated proof of principal that small molecule inhibitors of QseC-mediated signaling markedly inhibit the virulence of several pathogens in vitro and in vivo in animal models. This study utilized a high throughput screen to identify a potent small molecule, LED209, which inhibits binding of signals to QseC, preventing QseC's autophosphorylation, and consequently inhibiting QseC-mediated activation of virulence gene expression in enterohemorrhagic *E. coli* (EHEC), *Salmonella Typhimurium*, and *Francisella tularensis*. LED209 also prevented formation of lesions on epithelial cells by EHEC, and *F. tularensis* survival within macrophages. Moreover, LED209 treatment protected mice from lethal *S. Typhimurium* and *F. tularensis* infection. LED209 is not toxic and does not inhibit pathogen growth. Extensively, pharmacologic studies have been performed to first of all show that LED209 had not been cytotoxic in the animal models used in the study, such as mice and rabbits. This study demonstrated that inhibition of interkingdom intercellular signaling constitutes a novel and effective strategy for the development of a new generation of broad spectrum antimicrobial agents (Rasko et al. 2008).

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Chapter 13
The Role of the Microbiome in the Relationship of Asthma and Affective Disorders

Ana F. Trueba, Thomas Ritz, and Gabriel Trueba

Abstract The effect of stress, anxiety and other affective states on inflammatory conditions such as asthma is well documented. Although several immune pathway mechanisms have been proposed and studied, they cannot fully explain the relationship. In this chapter we present a new perspective on asthma development and exacerbation that integrates findings on the role of psychological factors in asthma with the microbiome and the hygiene hypothesis in asthma development.

Keywords Bacteria • Asthma • Atopy • Anxiety • Stress • Depression • Microbial endocrinology

13.1 The Human Microbiome

The animal bodies should be considered holobionts, consortia of symbiotic microorganisms (commensal and pathogenic) and animal (human) cells which have coevolved as a unique entity. Each component of the holobiont interacts chemically back and forth with the rest, and the health of the holobiont depends on the equilibrium between all of components (Relman 2012). Most microbiome members are colonizing the GI tract but others are present in the genitourinary and respiratory tracts (for reviews see: Hooper and Gordon 2001; Sommer and Bäckhed 2013; Lee and Mazmanian 2010). There are approximately 1000 bacterial species in the human microbiome and human cells constitute only the 10 % of the human consortium (Bäckhed et al. 2005). It is not surprising that many molecules circulating in animal tissues are of bacterial origin (Iyer et al. 2004).

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There are several bacterial species (e.g., members of the phyla Proteobacteria, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and *Cyanobacteria*) that thrive on all the surfaces that are exposed to the external environment including mucosal membranes (Hooper and Gordon 2001; Sommer and Bäckhed 2013). In the gastrointestinal tract the most common bacterial genera are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Clostridium* and *Lactobacillus* (Noverr and Huffnagle 2004). The microbiome is largely determined in the first year of life and remains fairly constant (Greer and O'Keefe 2010), but intestinal infections (pathogen colonization), antibiotic treatments and intestinal inflammation can alter its composition (Hoffmann et al. 2009; Sekirov and Finlay 2009; Lee et al. 2014).

The microbial cells produce large amounts of diverse molecules including neurotransmitters (such as GABA) and other molecules less studied, which have a profound effect in the development and function of human and animal tissues such as the immune system, GI tract (Sommer and Bäckhed 2013), and nervous system (Al-Asmakh et al. 2012; Collins et al. 2012; Sudo 2012; Forsythe and Kunze 2013; Douglas-Escobar et al. 2013, see Fig. 13.1a, b). The microbiota may also foment a healthy mucosal lining by promoting healing and blood supply. In addition, bacteria in the gut can induce blood vessel growth and aid in nutrient absorption (Greer and O'Keefe 2010). Microbial colonization of the gastrointestinal tract has an important role in the modulation and development of both systemic and localized inflammatory immune processes. The absence of some bacterial commensals or the lack of interaction with pathogens has been linked to chronic inflammatory conditions including atopy (Hooper and Gordon 2001; Sommer and Bäckhed 2013).

The holobiont paradigm is represented, to some extent, by the hygiene hypothesis (originally conceived by Strachan in 1989). This hypothesis proposes that human cells have co-evolved with pathogenic microorganisms (including infectious bacteria, human viruses, helminthes, etc., see Fig. 13.2). According to this hypothesis the interactions between human cells and pathogens are essential for a healthy immune system development. This implies that abnormalities in this interaction (such as excessive hygiene and lack of infections) in early life might be partially responsible for the development of immunologic diseases such as asthma, allergies and other inflammatory conditions later in life (Penders et al. 2007; Rook 2009; Sommer and Bäckhed 2013). This may explain the lower rates of allergic diseases in people from developing countries compared with industrialized ones. Research findings on the association of the microbiome and asthma complement the hygiene hypothesis, as both support the role of symbiotic (commensal and pathogenic) organisms on asthma development and exacerbation (Azad et al. 2013; Bendiks and Kopp 2013; Daley 2014).

Recent studies suggest that the composition of the microbiome might impact the development of the human stress systems including the hypothalamus–pituitary–adrenal axis (HPA axis; Sudo et al. 2004; Sudo 2012) and the adrenergic system (Goehler et al. 2005). This co-evolution has left signatures in microorganism genomes as evidence suggests that microbial pathogens can react to human stress hormones and thereby become more virulent and increase their growth rate (Bansal et al. 2007; Marks et al. 2013; Gonzales et al. 2013; Dickson et al. 2014). It is also possible that human hormones may alter the commensal members of the microbiome (Belay and Sonnenfeld 2002; Freestone et al. 2012).

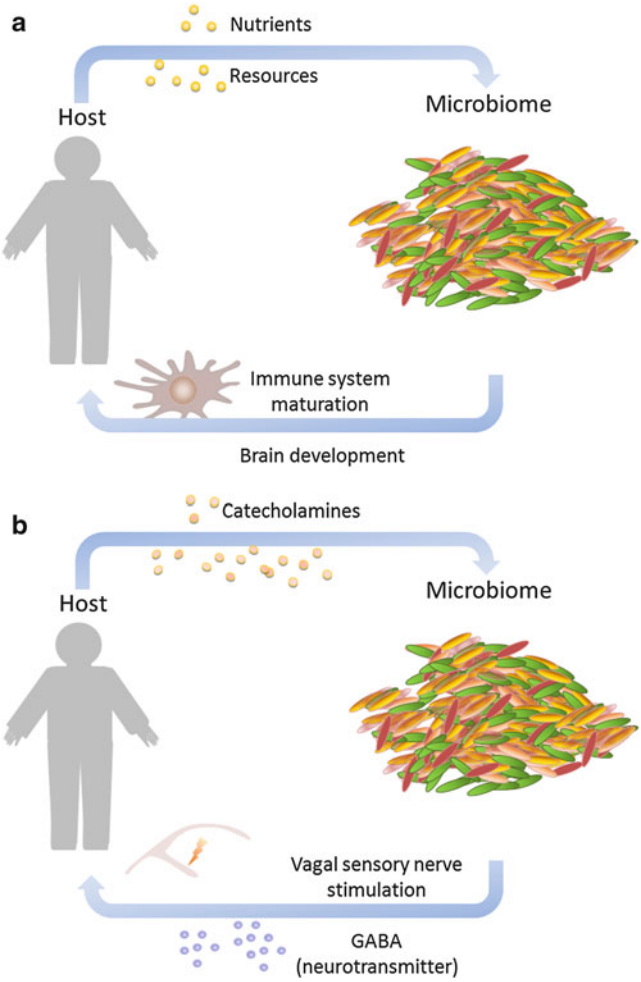

Fig. 13.1 (**a**) There is a symbiotic relationship between the microbiome and the host, as the host provides a habitat and resources including nutrients for the microbiome. In turn, the microbiome aids in the healthy development and functioning of animal tissues such as the immune system, GI tract, and nervous system. (**b**) The host and the microbiome communicate (interkingdom signaling) through catecholamines and neurotransmitters (such as GABA). The host's catecholamine production changes the proliferation of bacteria and in turn bacteria produce neurotransmitters (GABA) that alter the hosts' behavior

In this chapter we will explore interactions between psychological factors (anxiety, depression, and stress) and the composition of the microbiome and their links to development and exacerbation of asthma and allergies. We will begin by reviewing the relation between psychological factors and atopic diseases such as asthma. This includes the exploration of how early life stress might be involved in the development of psychological and inflammatory conditions, specifically allergic asthma. The effect of microbial colonization during early years of life on asthma and HPA

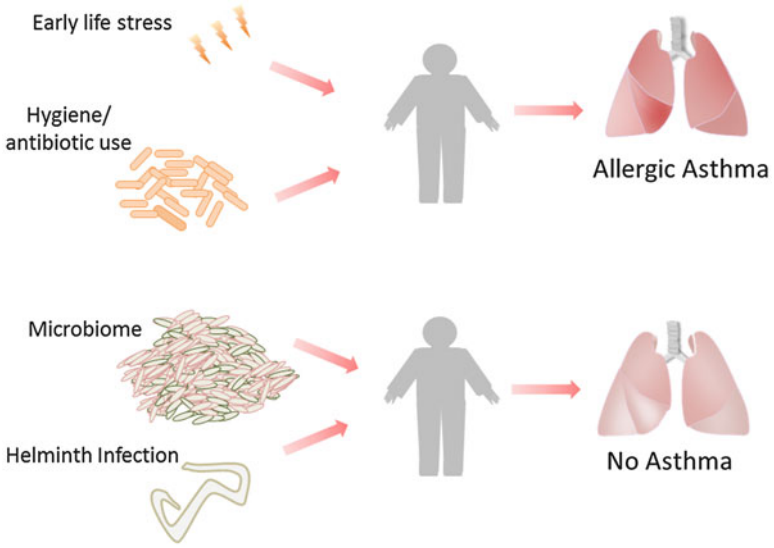

Fig. 13.2 Asthma development has been linked to early life stress and antibiotic treatment. Certain parasite infections (such as helminth infections) and the microbiome can provide protection against asthma

axis development will then be discussed. Following this we will consider some of the evidence for stress-induced alterations in bacterial behavior and will discuss the impact that this might have on asthma exacerbation. Finally, we assess future research directions and clinical implications involving the role of bacterial colonization, with potential avenues for novel interventions to ameliorate inflammatory and allergic conditions as well as psychiatric disorders.

13.2 Asthma

Asthma is a chronic disease of the airways. It is characterized by chronic inflammation, airway obstruction, airway remodeling, and increased mucus production, which result in intermittent symptoms of coughing, shortness of breath, wheezing, and chest tightness that vary in intensity across time (National Heart, Lung, and Blood Institute/National Asthma Education and Prevention Program [NHLBI/NAEPP], 2007). Approximately 300 million individuals suffer from asthma worldwide, making it one of the most prevalent chronic diseases among adults and the most common chronic diseases in children (Masoli et al. 2004). Asthma accounts for 1 % of disability-adjusted life years (DALYs) lost and its overall burden amounts to a loss of approximately 15 million DALYs per year (Bousquet et al. 2005).

The pathogenesis of asthma is complex because the disease can have a variety of underlying immune processes depending on the subtype (endotypes, Lötvall et al.

2011; Holgate 2002; Wenzel 2006). Atopic or allergic asthma has been characterized by elevations in type 2 T helper (Th2) cells (and Th2 cytokines IL-4, IL-5, IL13) relative to type 1 T helper (Th1) cells (and Th1 cytokines IL-2, IFN-γ). Th2 cells are involved in allergic processes and result in an excess of the antibody immunoglobulin E (IgE; Lötvall et al. 2011). Atopy prevalence can vary depending on the demographics including age, gender, and race. In the United States atopy accounts for 56.3 % of asthma cases in individuals that are 6–59 years old (Arbes et al. 2007). For young men with higher education levels living in metropolitan areas the prevalence of atopy can be as high as 74.1 %. Other asthma subtypes are characterized by inflammation that is caused by other immune cells such as natural killer (NK) cells, neutrophils, macrophages (Matangkasombut et al. 2009), bronchial epithelial cells, or dendritic cells (Lambrecht and Hammad 2003). For example, in a particular subtype, overactivity of NK cells can exacerbate allergic processes by producing Th2 cytokines. NK cells can also foment non-allergic asthma by causing airway hyperreactivity through reduction of regulatory T (T reg) cells and an increase in neutrophil recruitment by way of IL-17 production (Matangkasombut et al. 2009). Other asthma subtypes are non-eosinophilic and are characterized by Th1 over expression (Zhu et al. 2010). This review will focus mostly on atopic asthma over other subtypes.

There is a high prevalence of psychological disorders among patients with asthma. Associations have been found between asthma and anxiety symptoms (Slattery and Essex 2011) and several anxiety disorders (Goodwin et al. 2004; Lavoie et al. 2011). Depression prevalence also appears to be higher among asthma patients (Slattery and Essex 2011; Wong et al. 2013), although the literature is more variable (Opolski and Wilson 2005). Negative emotions or mood have been linked to reduced airway function (Ritz et al. 2013) or worsening of asthmatic symptoms (Rietveld et al. 1999), as have depression (Richardson et al. 2006), adverse life events (Sandberg et al. 2000), low socioeconomic status, and stress (Chen et al. 2010).

Stress-induced changes in immune function have been proposed as important mechanisms that could explain asthma symptom worsening in the context of psychosocial stress (Kang et al. 1997; Liu et al. 2002; Chen et al. 2010; Trueba and Ritz 2013). A substantial number of studies suggest that the association between inflammation and psychopathology can start early in life. Studies have examined the impact of adversity and mental health in childhood on subsequent allergic condition development. Epidemiological studies have shown an association between a history of asthma and anxiety disorders in children (Ortega et al. 2002) and adolescents (Slattery and Essex 2011). Prospective longitudinal research has shown a relationship between early life adversity or young adulthood anxiety disorder and the subsequent the development of asthma (Hasler et al. 2005; Scott et al. 2008) Research also suggests that maternal stress in early life (from birth until age 7) is associated to increased likelihood of childhood asthma (Kozyrskyj et al. 2008). Although there are several proposed immune mechanisms, much remains to be learned about the pathways underlying the association between life stress and subsequent asthma development.

It has been suggested that the effect of stress on immune processes can start as early as in the intrauterine phase (Wright 2010; Wright et al. 2010). Indeed, studies have found that maternal psychological stress during gestation was associated with

greater likelihood of asthma in childhood (Cookson et al. 2009). High levels of umbilical cord immunoglobulin E (cIgE; an allergy-related immunoglobulin) have been associated with family and maternal history of atopic conditions and studies have found that psychosocial factors such as stress are related to elevated cIgE levels (Lin et al. 2004). Similarly, the Urban Environment and Childhood Asthma Study examined 557 families and found that prenatal maternal stress (e.g. violence, financial difficulties) was related to an increase in a asthma-related Th2 cytokine (IL-13) release by cord blood mononuclear cell (CBMC) (Wright et al. 2010). This finding suggests that early stress heightens asthmatic immune responses in perinatal infants and this could prime infants to develop asthma (Wright 2010).

13.3 Asthma and the HPA Axis

A characteristic that asthmatics and individuals with psychopathology seem to have in common are abnormalities in their physiological response to stressors. There is evidence to suggest that stress system malfunctioning is related to the development of asthma (Buske-Kirschbaum et al. 2003; Buske-Kirschbaum and Hellhammer 2003; Wright 2007; Priftis et al. 2008) and psychopathology (Glover et al. 2010; Glover 2011).

The HPA axis is one of two important systems that modulate physiological responses to stress. The end result of the HPA axis activation in response to stress is the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland (Webster et al. 1998) and glucocorticoids from the adrenal cortex (Elenkov and Chrousos 1999). The other stress system is the locus ceruleus-norepinephrine (LC-NE)/sympathetic nervous system, which mediates the fight-or-flight response. Activation of the sympathetic nervous system releases norepinephrine from presynaptic terminals at organs and glands. In addition, the adrenal medulla releases catecholamines into the blood stream when stimulated by sympathetic nerve terminals (Elenkov et al. 2000).

Previous research has found an association between HPA axis dysregulation and allergic asthma. One study observed that children with allergic asthma compared to controls had a blunted cortisol response when exposed to standard laboratory stressor (free speech and mental arithmetics under evaluative threat). None of these children were on corticosteroid medications (Buske-Kirschbaum et al. 2003). Similarly, children with allergic dermititis also have showed blunted cortisol responses to this type of laboratory stressor (Buske-Kirschbaum et al. 1997) and these same findings have been replicated in adolescents with atopic disorders (Wamboldt et al. 2003) and adults with asthma (Buske-Kirschbaum et al. 2002). There is also evidence that the circadian rhythm of cortisol in infants at risk for allergic diseases (defined by having parents with allergic disease) is flattened such that they have a reduced morning cortisol response and lower diurnal cortisol release (Ball et al. 2006). Studies have found that this altered HPA axis response is independent of corticosteroid use.

Indeed, there is some evidence that use of cortisosteriods could an actually reverse the adrenal suppression that observed in asthmatics display (Priftis et al. 2006, 2008). In drawing conclusions from these findings it is important to consider the complexity of asthma with different subtypes (endotypes, see above) each of which involve distinct immune processes. Most of the literature in this area has focused on dysregulation of the HPA axis and on atopic conditions, which means that findings might not generalize to all asthma subtypes.

One potential mechanism that could explain the altered HPA axis responses in asthma and atopy is an elevation in circulating inflammatory cytokines. Elevations in interleukin 1 (IL-1) can lead to the release of glucocorticoids such as cortisol, as IL-1 stimulates corticotropin-releasing hormone (CRH) release which initiates a cascade of HPA axis activation (Glaser and Kiecolt-Glaser 2005). This relationship is bidirectional, such that hyperactivity of the HPA axis can lead to glucocorticoid resistance, which can result in exaggerated inflammatory processes (Raison et al. 2006) and a shift towards Th2 immune responses (Van Lieshout et al. 2009), which results in asthma exacerbation.

13.4 HPA Axis and Psychopathology

The role of early life stress in the development of both allergic conditions and psychopathology can be attributed in part to alterations in the development of the HPA axis. There is evidence to suggest that there is a critical period of HPA axis development. Abnormalities in the development of the HPA axis during this period might result in permeant dysregulation associated with the development of anxiety symptoms, conduct disorder, or attention deficit hyperactive disorder (ADHD) in adult age (Glover 2011). In a substantial portion of cases of clinical depression the HPA is hyperactive, in that about half of the patients with major depression have heightened cortisol levels (Pariante and Lightman 2008),increased CRH levels in the cerebrospinal fluid, and elevations in CRH messenger RNA and protein in structures of the limbic region (Forsythe et al. 2010). The same study found that about half of depressed patients do not show the normal drop in cortisol levels after being administered dexamethasone, a challenge that typically results in a suppression of cortisol release because dexamethasone is a negative feedback signal for the pituitary gland that should result in a decreased secretion of ACTH and cortisol. Patients also did not respond to the dexamethasone-CRH test (which is more sensitive for major depression than dexamethasone alone), in which dexamethasone is administered at night and CRH injected on the subsequent day in-between cortisol and ACTH measurements (Heuser et al. 1994). Both findings suggested that the negative feedback by glucocorticoids is blocked. This may be caused by a reduction in the number and sensitivity of glucocorticoid receptors expressed by organs and immune cells including those involved in asthma (Pariante and Lightman 2008).

13.5 Intestinal and Airway Microbiomes in Asthmatics

The microbiome residing in the gut and the respiratory tract of asthmatics is distinct from that of healthy individuals. There is some evidence to suggest that infants who develop allergies have a different bacterial composition in their gut than those that are not allergic (Kirjavainen et al. 2001; Björkstén et al. 2001; Ouwehand et al. 2001). Studies have also shown that individuals with asthma have a distinct microbiome in their respiratory tract compared to that of healthy individuals (Hilty et al. 2010; Huang et al. 2011). Using 16S-rRNA genes to determine the bacteria that reside in the airways, Hilty et al. (2010) found larger proportions of Proteobacteria, a phylum that contains potential pathogens such as *Haemophilus*, *Moraxella* and *Neisseria* spp., in the airways of asthmatics in comparison with healthy individuals. Yet another study found that asthmatic airways have a greater diversity of bacterial species. Furthermore, certain bacterial families such as Sphingomonadaceae or Oxalobacteraceae that reside in the airways of asthmatics have been associated with airway hyperresponsiveness (Huang et al. 2011). Taken together, this research suggests that asthma pathogenesis and/or exacerbation might be affected by microbial composition of airway microbiome. However, cause and effect have not been established and there is also the alternative possibility that chronic inflammation of airway mucosa changes the microbiome (Sekirov and Finlay 2009).

13.6 Microbiome, Pathogens and Asthma

Some studies (including meta-analyses) support the hygiene hypotheses as they suggest that the lack of particular infections in early life may be linked to higher predisposition to asthma (Leonardi-Bee et al. 2006; Penders et al. 2007). The nature of the protective mechanism conveyed by infections are not fully understood, as they may constitute direct interactions of the pathogens with human cells, or involve indirect pathways though intestinal infections that also cause changes in the microbiome (Sekirov and Finlay 2009). Asthma and other atopic conditions are more prevalent in developed countries than in developing countries which often differ in the extent to which they emphasise hygiene (Wills-Karp et al. 2001). Another relevant study found that children who went to day care and lived in households with older siblings suffering from recurrent infections were less likely to develop atopic conditions (Ball et al. 2000). In a more recent study, Lynch and colleagues (2014) found that lack of or rare occurrence of exposure to bacteria and allergens in the first 3 years of life made children more likely to develop atopic conditions and wheezing. The main evidence that some microbes can reduce the possibility of developing asthma is associated with research on the hygiene hypothesis. Some examples are infections caused by hookworms (Leonardi-Bee et al. 2006) *Toxoplasma gondii*, *Helicobacter pylori*, *Mycobacteria tuberculosis*, hepatitis A that have been associated with reduced likelihood of developing asthma or allergies (Umetsu et al. 2002). The

immune mechanism proposed to underlie the hygiene hypothesis is mostly centered around the effect of microorganisms on the Th1/Th2 balance. Among atopic asthmatics there is imbalance between Th1 and Th2 cells, in that there is an increase in Th2 cells that leads to allergic inflammation in the airways (Mosmann and Coffman 1989). Infections caused by specific pathogens and colonization of certain bacterial species may help balance Th1 and Th2 cell proliferation, by increasing the production T regulatory cells (Treg cells), which protects against atopic asthma development by preventing overproduction of Th2 cells (Umetsu et al. 2002; Macpherson and Harris 2004; Rook and Lowry 2008). However, new perspectives propose that specific microorganisms may have an important role in the maturation of the mucosal and innate immune system. Some bacteria can lead to mucus production (induced by IL-13) as well as induce an increase in IgA levels, both of which protect and strengthen the epithelial barrier (Cookson 2004). It has been proposed that the maturation of the immune system induced by infection is a process that occurs in early life, but findings suggest that some infections continue to be immunomodulary in adults, by causing and mucosal immune "tolerance" towards antigens (Umetsu et al. 2002). This perspective on the hygiene-asthma relationship involving maturation of mucosal and innate immune systems pertains more to development of other asthma subtypes that are not associated with Th2 overexpression but with overexpression of NK cells, neutrophils, or macrophages (Matangkasombut et al. 2009).

The intestinal microbiome has also been linked to asthma. There is some evidence to suggest that infants that who develop allergic asthma have different bacterial composition in their gut than those that are not allergic (Ouwehand et al. 2001; Kirjavainen et al. 2001; Björkstén et al. 2001). The microbiome and the immune system have a complex relationship which may be central to the understanding of asthma pathogenesis. As mentioned above there are many bacterial species including pathogens that colonize and infect the human intestines that have an important role in the healthy development and function of the human immune system (Hooper and Gordon 2001; Sommer and Bäckhed 2013; Round and Mazmanian 2009).

Microbiome disturbances caused by antibiotic treatment during first year of life may predispose to asthma (Droste et al. 2000; Murk et al. 2011). Furthermore, infants born by caesarean section and thereby not exposed to the mother's gut microbiome) are 20 % more likely to develop asthma (Thavagnanam et al. 2008). The specific intestinal microbiome composition that is related to allergic asthma has not been well defined. Several studies suggest that the *Bifidobacteria* have a significant role in the development of atopic/allergic conditions. More specifically, infants with allergies tend to have reduced concentrations of *Bifidobacterium bifidum* (Ouwehand et al. 2001; Umetsu et al. 2002). Similarly, there is evidence that infants with atopic eczema have lower levels of *Bifidobacteria* and Gram positive organisms (Kirjavainen et al. 2001). It appears that this specific intestinal microbiome profile is associated with allergic conditions regardless of the country of origin. One study found that infants from Estonia and Sweden who developed allergies by the age of 2 had lower concentrations of bifidobacteria and enterococci in their first year and higher levels of clostridia at 3 months and *S. aureus* at 6 months (Björkstén et al. 2001).

Some findings caution interpretations drawn from experiments of the microbiome and asthma (as is valid for other diseases). Contradictory findings on beneficial versus detrimental effects of infections make it especially challenging to ascertain which bacteria specifically increase the likelihood of developing asthma and which ones decrease it. It seems that several factors might determine if the colonization or infections from a particular bacterial strain is protective or harmful. This includes the age of the host at the time of contact with the bacteria and the amount of bacterial proteins and endotoxin produced by the bacteria (LPS specifically; Zhu et al. 2010). LPS has been shown to have protective effects if exposure is early in life but detrimental if the host is older. LPS is produced by several gram-negative bacteria (e.g., *E. coli* bacteria), some of which are associated with protective effects such as *Helicobacter pylori* (Umetsu et al. 2002). Research suggests a time- and dose-dependent relationship: excessive LPS exposure may lead to increased allergen sensitization (Schröder 2009) but early life exposure to LPS protects against atopy (Zhu et al. 2010). Furthermore there is significant variability in the genomes of bacteria. Two bacteria that are of the same species can have very different genes, which ultimately can change the specific interaction that these bacteria have with human cells. These genomic differences could also explain some of the contradictions about the protective versus detrimental effects of bacteria on asthma (Gogarten et al. 2002).

Studies suggest that certain bacterial pathogens that colonize throats of young infants can lead to wheezing, these include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* (Bisgaard et al. 2007) and *Chlamydia pneumoniae* (Korppi 2010). One study infected mice with *Chlamydia pneumoniae* subsequently exposed to human serum albumin (HSA) and the infection triggered increased sensitization toward HSA (Schröder et al. 2008).

Some microorganisms promote the mucosal immunity by stimulating mucosal lymphocytes to produce IgA (Cookson 2004), but colonization or infection by other bacteria can actually degrade IgA (Kilian et al. 1995). Certain bacterial species colonizing the respiratory tract produce specific proteases that cleave IgA. These include: *Streptococcus mitis biovar 1*, *Haemophilus influenzae*, *Haemophilus parahaemolyticus*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*. Cleavage of IgA is more prevalent in children with a history of atopic conditions than in non-atopic children. This may suggest that IgA1 cleavage by bacterial proteases can contribute to the development of immunological dysfunctions in the mucosa (Kuklinska and Kilian 1984) related to atopy. One study found evidence of elevated levels of IgA1 protease-producing bacteria in the oropharynx of 18-month-old atopic infants specifically *Streptococcus mitis biovar 1* (Kilian et al. 1995). Stress may play an interesting role in this relationship between bacteria and IgA levels. While a number of studies has shown increase in IgA levels following acute stressors (e.g., Bosch et al. 2002, 2003; Brandtzaeg 2007; Trueba et al. 2012) other studies have shown that chronic stressors can lead to a decrease in IgA titers (Henningsen et al. 1992; Drummond and Hewson-Bower 1997). This adds to the complexity involved in the relationship of bacterial contact and asthma (Zhu et al. 2010).

Many of the studies examining the relationship between bacterial infection and asthma have probably recruited a mixture of asthmatic subtypes (Zhu et al. 2010).

The hygiene hypothesis postulates specific changes in Th1/Th2 balance dependent on contact with pathogens and infections in early life that lead to protection against atopic conditions. With only about half of the asthma cases being atopic (Holgate 2007) it may be that studies suggesting an increase in the likelihood of asthma development as a result of bacterial infection have involved samples of asthmatics with a substantial number of non-atopic cases.

Regardless of whether the lack of gut bacterial colonization or repeated respiratory infection is related to asthma development, as mentioned above, it appears that there is a relationship between certain bacterial species and asthma (Hilty et al. 2010; Huang et al. 2011). One alternative interpretation of some of these findings is that asthmatic individuals exhibit abnormalities in their mucosa, which changes the microenvironment and bacterial colonization that occurs in their airways. It may also be the case that at an early stage of development the airway epithelium of asthmatics differs from that of healthy individuals and allows for the colonization of different bacterial species. More longitudinal studies of mucosal characteristics and bacterial colonization are needed to elucidate the direction of their relationship.

13.7 The Role of the Microbiome in the Development of Stress Responses and Potential Affective Disorders

New imaging technologies have revealed massive and direct connections between the gastrointestinal tract and the brain (Bohórquez et al. 2014; Tillisch et al. 2008). Recent research also suggests that the intestinal microbiome is involved in the development of the brain (for reviews see: Al-Asmakh et al. 2012; Douglas-Escobar et al. 2013; Collins et al. 2012; Sudo 2012), including the systems that modulate adaptive responses to stress, the HPA axis (Sudo et al. 2004) and the autonomic nervous system (Goehler et al. 2005). This could suggest that the microbiome is implicated not only in the development of asthma, but also in the pathogenesis of affective disorders. Abnormal bacterial colonization is yet another novel mechanism that could explain the comorbidity of asthma and psychopathology (see Fig. 13.3).

13.7.1 HPA Axis and the Microbiome

Recent research suggests that bacteria can determine the set-point of the HPA axis (Sudo 2012). The authors compared germ free mice with another group that had gut microbiota, but were pathogen free. They found that restraint stress (immobilization for 1 h) in mice caused an exaggerated ACTH and corticosterone elevation in the germ free mice compared to those with microbiome and pathogen free (corticosterone is the equivalent of cortisol in animals, the main glucocorticoid released in response to stress by the adrenal glands in animals). This was reversed when these mice where given bacteria from the feces of the pathogen free mice and even more

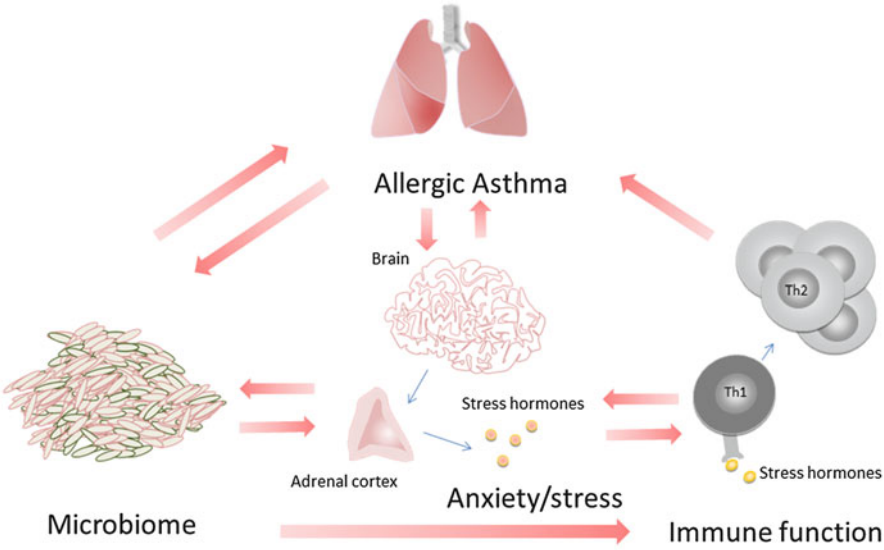

Fig. 13.3 The relationship between affective disorders and asthma is complex and bidirectional. Stress can cause changes in immune function that can result in asthma exacerbation. In turn, the increase in certain cytokines such as IL-1 can cause an increase in cortisol (stress hormones). The microbiome can alter anxiety behavior and in turn the presence of catecholamines (stress hormones) can change bacterial proliferation. The microbiome has also been associated with the development of allergic asthma as it can modulate immune processes relevant in allergic asthma. Hypothetically, stress can also induce changes in the airway bacterial colonization and thereby facilitate inflammatory processes linked to allergic asthma, which in turn may further alter airway microbiome colonization

so when they were given the bacterial species *B. infantis* (Sudo et al. 2004). Interestingly, this reversal in the HPA axis responsiveness was only achieved if the bacteria were administered early in the mice development, specifically, only before the age of 6 weeks. The beneficial effects were not observed in mice that were past 14 weeks old. This highlights a possible developmental window that may be particularly relevant for bacteria colonization. The study also indicates that certain bacterial species have a stronger effect on the HPA axis than others. In partial support of these findings, Neufeld et al. (2011) also observed elevations in corticosterone in the serum of mice there were germ-free.

Bacteria can also modulate stress responses by causing alterations in the autonomic nervous system activity. One study found that oral delivery of bacteria *Campylobacter jejuni* (Gaykema et al. 2004) or *Citrobacter rodentium* resulted in stimulation of vagal sensory nerves and the central autonomic network (Goehler et al. 2007, see Fig. 13.1b). The mechanism through which bacteria are thought to activate the vagal neurons is through the increased expression of c-Fos protein. C-Fos is a transcription factor considered a marker of neuronal activation as its expression is associated with neuronal firing (increased number of action potentials). There is evidence to suggest that bacterial LPS induces the expression of

c-Fos protein in neurons located in the circumventricular organs (structures that lack a blood–brain barrier), the brainstem and pons (Goehler et al. 2005). Similarly oral administration of live bacteria causes an increase in the c-Fos expression in vagal sensory neurons (Goehler et al. 2005). C-Fos expression also increased in the paraventricular nucleus (PVN) of the hypothalamus 6 h after inoculation of *B. infantis* and this elevation occurred before increases in cytokines were observed (Sudo et al. 2004). In another study rats infected with *Salmonella enterica*, serovar Typhimurium increased c-Fos protein expression in the PVN and superaoptic nucleus. When these mice were subjected to a subdiagphragmatic vagotomy it reduced the c-Fos expression induced by *Salmonella enterica* serovar Typhimurium (Wang et al. 2002). These findings suggest that certain bacteria can activate the vagal sensory nerves and therefore may modulate autonomic nervous system activity.

13.7.2 Microbiome and Anxiety Behaviors

The *in vitro* studies presented in the previous section may imply that bacteria can also be impacted by human stress responses (Belay et al. 2003; Green et al. 2003; Freestone et al. 2007a, b; Diard et al. 2009). Interestingly, recent observations suggest that this relationship is bidirectional, as there is evidence suggesting that bacteria influence autonomic nervous system and HPA axis reactivity which can result in changes in anxiety levels and eating behavior (Alcock et al. 2014, see Fig. 13.3). There is evidence that bacteria can induce cravings for certain food (Alcock et al. 2014). In the fruit fly (*Drosophila melanogaster*) the bacteria *Lactobacillus plantarum* in the gut can determine mate selection (Sharon et al. 2010). There is other evidence from animal studies that gut microbiota can also modulate inflammatory pain responses. Amaral et al. (2008) found that germ-free mice had reduced pain measured by recording the hindpaw flexion reflex in response to inflammatory pain. Studies in mice also suggest that bacteria modulated psychological characteristics of the host, such as anxiety behavior (Neufeld et al. 2011; Heijtz et al. 2011). One study found that mice free of bacteria exhibited no anxiety behaviors compared to those that had some bacteria but no pathogenic species (Heijtz et al. 2011). The presence of certain bacteria altered hormone levels such as adreno-corticotrophic hormone (ACTH; Sudo et al. 2004). Germ-free mice have also been found to have elevated noradrenaline, dopamine and serotonin in their striatum when compared to mice that had no pathogenic germs (Heijtz et al. 2011). Another study compared germ free mice and mice that were free of pathogenic bacteria (Neufeld et al. 2011) and found that germ free mice exhibited less anxiety behavior than mice that had a pathogen-free microbiome. The altered behavior of the germ free mice was also accompanied by an increase in brain-derived neurotrophic factor (BDNF) and a decrease in serotonin 1 A receptor expression in the hippocampus. These results suggest that gut microbiome causes changes in anxiety behavior which are linked to alterations in neuronal processes at a molecular level. This also elucidates some of the molecular mechanisms underlying the relationship between gut bacteria and host behavior.

Some research has found that the striatum of germ-free mice, compared to normal mice, has higher levels of synaptophysin and PSD95, which are proteins that have an important role in synaptogenesis (Heijtz et al. 2011). Yet another possibility is that anxiety behaviors might be augmented through increases in certain cytokines that are produced as a result of abnormal bacterial colonization. Specifically, there is evidence that bacterial endotoxin and/or IL-1 can increase serotonin in the hippocampus and the medial prefrontal cortex in mice (Rook and Lowry 2008).

One important distinction worth noting is that germ-free mice are distinct from mice that have had dysbiosis, in which the composition of bacteria changes as a result of antibiotic treatment or infection. Studies suggest that mice with dysbiosis (caused by infections by pathogenic bacteria) that had to be treated with antibiotics showed an increase rather than decrease in anxiety behaviors (Lyte et al. 2006; Bercik et al. 2011). A study conducted by Lyte et al. (2006) suggests that anxiety behaviors caused by pathogenic bacteria *Citrobacter rodentium* might be mediated by vagal sensory nerve stimulation. Another study found that innoculating mice that had an altered gut epithelial barrier with human *Bacteroides fragilis* (a commensal bacteria) reduced abnormal sensorimotor behaviors and anxiety (Hsiao et al. 2013). Research overall suggests that bacteria interact with both the hormonal stress and the immune system, therefore changing the dynamics between stress and immune processes, with potential consequence for host behavior and inflammatory conditions such as asthma.

13.7.3 Microbiome and Depression

There is evidence that the microbiome can possibly modulate depressive symptoms (Wang and Kasper 2014). The presence of certain bacteria (such as Proteobacteria) can be related to dysphoria, which may have the function of motivating the host to eat certain foods that favor their proliferation (Alcock et al. 2014). Memory impairment, which is observed in individuals with major depressive disorder (Snyder 2013), could also be linked to changes in commensal bacteria (Gareau et al. 2010). There have been several studies showing that infections in general can induce symptoms of depression including lethargy, loss of energy, difficulty concentrating, and decreased appetite among others, as a result of the sudden increase in inflammatory cytokines (Maes 1995; Dantzer et al. 2008). Bacteria could also modulate depression through disruptions in the HPA axis responses as detailed above (Forsythe et al. 2010).

Depression is characterized by disruptions to the serotonergic system (Ogilvie et al. 1996) and alterations in BDNF expression (Martinowich et al. 2007). Both serotonin (Forsythe et al. 2010) and BDNF expression can be modulated by certain bacteria (Sudo et al. 2004; Bercik et al. 2011). One study found that germ free mice that were administered probiotics had a threefold increase in serotonin (Forsythe et al. 2010). There is also evidence that the germ free mice have a decrease in serotonin 1 A receptor expression in the hippocampus (Neufeld et al. 2011). Furthermore germ free mice showed a reduction in BDNF in the hippocampus

and cortex (Sudo et al. 2004). In another study, the administration of antimicrobials to specific pathogen free mice changed gastrointestinal microbiota, increased BDNF expression in the hippocampus, and increased exploratory behavior (Bercik et al. 2011). There is also evidence that norepinephrine and serotonin levels in the cortex and hippocampus are reduced in mice that are germ-free compared to mice with microbiome but free of a specific pathogen (Forsythe et al. 2010). Intestinal bacteria produce GABA, which is known to interact with the serotonergic and BDNF systems and is linked to depression and anxiety symptomatology (Kalueff and Nutt 2007). Lactobacilli in particular are among the bacteria that produce GABA (Forsythe et al. 2010) and when ingested it can modulate GABA receptor expression through the vagus nerve (reducing them in the prefrontal cortex and amygdala, and increase in the hippocampus; Bravo et al. 2011).

Like depression, asthma is also associated with changes and disruption of several neurotransmitter pathways, specifically serotonin (Lechin et al. 2002) and BDNF (Nockher and Renz 2006). However, it seems that serotonin levels in asthmatics are high, which is in direct contrast to depressed individuals who have low serotonin levels. These abnormal levels might be related to abnormal activation of the NFkB pathway (in both asthma and depression). In depression there is an increased activation in NFkB and in atopic conditions NFkB might be inhibited by glucocorticoid use. However, interestingly there is evidence that a selective serotonin reuptake inhibitor may reduce asthma symptoms (Van Lieshout et al. 2009). BDNF levels are altered in both asthma and depression, however, asthmatics express high levels of BDNF (Nockher and Renz 2006) in contrast individuals with depression who express low levels (Lechin et al. 2002). One reason for these discrepancies could be that BDNF levels in patients with depression are usually determined centrally whereas in asthmatics BDNF is measured in the airways. There are many other pathways that could serve to explain the relationship between asthma and depression, such as aberrant levels of different cytokines (IL-1ß and TNF-α), prostaglandins, neuropeptides (such as tachykinins) among many others (Van Lieshout et al. 2009). The observed instances of microbiome involvement in the modulation of key neurotransmitters suggest that host-microbiome interactions are important modulators of neutrotransmitter and neuromodulator expression relevant to asthma and depression, although their contribution to development and/or exacerbation of theses conditions is not yet ascertained. Further research on the role of bacterial colonization in the comorbidity of depression and asthma is therefore warranted.

13.8 Microbial Endocrinology and Asthma Exacerbation

Some bacteria are known to have the ability to sense host stress hormones; some of the most direct evidence came from studies carried out in pathogens and opportunistic pathogens which have been shown to have receptors and signal transduction systems allowing them to respond to animal stress hormones (such as norepinephrine) and increase their virulence (Lyte et al. 1996, 2011; Belay and Sonnenfeld

2002; Bansal et al. 2007; Freestone et al. 2007b; Hughes and Sperandio 2008; Freestone et al. 2012; Gonzales et al. 2013; Marks et al. 2013; Dickson et al. 2014; see Fig. 13.1b).

There is evidence that bacteria residing specifically in the respiratory tract are also susceptible to stress hormones. *Streptococcus pneumoniae*, a pathogenic bacteria that can cause infection of the airways, proliferates in the presence of norepinephrine (Dickson et al. 2014). It frequently colonizes the mucosa surface (up to 40 % of individuals) creating a biofilm and despite the low infection rate it is leading cause of bacterial respiratory infections. One study found that the elevations in norepinephrine as a result of influenza A virus infection, lead to the release of *Streptococcus pneumonia* bacteria from biofilm allowing these bacteria to spread to other mucosal surfaces. Interestingly, these dispersed bacteria had a unique virulence gene expression that made them more able to cause infection in germ-free sites including the blood stream. This effect was observed both *in vitro* and *in vivo* (Marks et al. 2013).

Opportunistic bacteria residing in the airways such as *Pseudomonas aeruginosa* (Freestone et al. 2012) and *Klebsiella pneumoniae* also proliferate in the presence of norepinephrine and dopamine. They also grow when exposed to epinephrine and isoproterenol but to a lesser degree (Belay and Sonnenfeld 2002). It is unusual for *Pseudomonas aeruginosa* to cause pneumonia in healthy individuals, but this bacterial species is responsible for many cases of pneumonia in patients that are in intensive care as they are using ventilators. Interestingly, one study found that catecholamine inotropes, a medication that is frequently prescribed to patients in intensive care, can increase the proliferation of these bacteria, increasing the bacterial count by 50 fold. The administration of catecholamines can also cause the proliferation of *Pseudomonas aeruginosa* biofilm and can make the biofilm more virulent and toxic when in contact with airway epithelia (Freestone et al. 2012).

13.9 Microbial Endocrinology, Psychoneuromicrobiology and Gut Microbiome

Microbial studies, using culture dependent and culture independent techniques, suggest that the microbiome may be also influenced by host stress hormones such as norepinephrine. Research suggests that catecholamines could also change the proportions or composition of commensal bacteria in the mucosal surfaces of the gut (Bailey et al. 2010, 2011) and respiratory tract (Dickson et al. 2014). Indeed, early studies in this field found that infant rhesus monkeys, when exposed to psychosocial stressors such as separation from the mother, suffer a significant decrease in intestinal lactobacilli (Bailey and Coe 1999). There have been similar findings in humans (Knowles et al. 2008). One study collected fecal samples from students during exam week and during a non-stress week and found that there was a significant decrease in lactic acid bacteria during the stress period (Knowles et al. 2008). Interestingly, reductions in *Lactobacillus* have been previously associated with atopic development

in infants (Björkstén 2004). Studies like this have significant limitations, in that the bacteria counts were determined using cultures, and only 1 % of the bacteria residing in the gut can be cultured *in vitro* as most intestinal bacteria are unculturable (Nocker et al. 2007). High throughput sequencing of microbiome DNA from stressed (through social disruption) and non-stressed mice showed significant reduction in the genus *Bacteroides*, and elevation of the genus *Clostridium* in the stressed group in their gut. Interestingly, the social stressor was also associated with elevations in inflammatory cytokines and chemokines, specifically IL-6 and MCP-1. The increase in these molecules may have been related to the changes in bacteria genera, specifically *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea* (Bailey et al. 2011).

From the research presented in this section, it is likely that stress might change not only the composition of the human microbiome but also may increase virulence in some pathogens of the respiratory tract. Both changes could lead to asthma exacerbation.

13.10 Probiotics and Fecal Transplants Reduce Inflammation and Possibly Anxiety Symptoms

The connections between the microbiome and asthma, atopic diseases, and psychological phenomena has prompted scientist to explore the possibility of using probiotic products in the treatment of these diseases. Specifically, studies have focused on probiotics of bacterial species such as *Bifidobacterium* spp. and *Lactobacillus* spp. (Forsythe et al. 2007), which have been associated with beneficial effects on immune and inflammatory processes (Feleszko et al. 2007).

A systematic review of randomized controlled trials found that probiotics can reduce asthma symptoms and medication use (Vliagoftis et al. 2008). One study found that the leukocytes of participants that consumed yogurt (16 oz) had increased IFN-γ production and IL-2 (Th1 cytokines) after being stimulated with the mitogen phytohaemagglutinin (PHA) (Singh and Ranjan Das 2010), which can reduce Th2 immune processes involved in allergic asthma. Probiotics have been reported to enhance the phagocytic activity of blood polymorphonuclear leukocytes (BPMC), which entails the engulfing of foreign molecules or microorganisms by immune cells (Singh and Ranjan Das 2010). One randomized control trial found that prophylactic treatment with probiotics reduced the likelihood of pneumonia compared to the placebo (Morrow et al. 2010).

Probiotics may modulate the organism's distress symptoms. One study found patients with chronic fatigue syndrome that took a probiotic had a significant increase in their *Bifidobacterium* spp. and *Lactobacillus* spp. and a reduction in their anxiety and depression symptoms compared to the group taking the placebo (Rao et al. 2009). Another study found that the daily intake of probiotics (*Lactobacillus helveticus R0052* and *Bifidobacterium longum R0175*) reduced anxiety behavior in rats and in human participants they found reduced depressive and anxious mood, and urinary cortisol. In addition, in human participants probiotic

administration reduced median urinary cortisol (but not overall cortisol levels; Messaoudi et al. 2011). One study found that female participants who ingested fermented milk with *Bifidobacterium animalis subsp Lactis*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, *and Lactococcus lactis* subsp. *lactis* had reduced responses in the areas of the brain (measured using functional magnetic resonance imaging) associated with to emotional processing (primary interoceptive and somatosensory regions; Tillisch et al. 2013) when exposed to emotional faces. Ingestion of *Lactobacillus rhamnosus* decreased anxiety and depression behavior in mice another study and there are indications that the sensory vagus nerve mediated this effect (Bravo et al. 2011). However, randomized control trials examining effects of probiotics in individuals with clinical anxiety are needed to determine the extent of effects probiotics can have on human anxiety symptoms.

Despite the large number of reports of positive outcomes from probiotic use, some meta-analyses have shown no beneficial effects of probiotics in children with asthma (Boyle and Tang 2006; Azad et al. 2012; Elazab et al. 2013). One meta-analysis found that probiotic use can reduce IgE levels in children (indicating a reduced atopy) but had no effect on asthma symptoms including wheezing (Elazab et al. 2013). Meta-analytic evidence for the use of probiotics to prevent or reduce allergic diseases is also weak (Osborn and Sinn 2007).

The mechanism of action of probiotics, which is thought to be colonization of the intestines, has been challenged by studies showing that bacteria adapted to grow under industrial conditions are unable to colonize human intestines (McNulty et al. 2011). Fecal transplant treatment, in which fecal bacteria from a healthy organism is transferred to another organism, might be a better method to increase bacterial diversity in the gut because these bacteria have not lost their ability to survive and grow in the human intestine (Damman et al. 2012). There is evidence that fecal transplants can be used to treat infections from *Clostridium difficile* (Bakken et al. 2011). There is also preliminary evidence that fecal transplants might help in the treatment of metabolic and gastrointestinal disorders (Damman et al. 2012).

Overall, the understanding of the interactions between microbiota and human diseases is still in its infancy and some researchers have recommended exercising caution in extrapolating to humans from work carried out in mice (Hanage 2014; Viswanathan 2014).

13.11 Concluding Remarks and Future Directions

In this chapter we sought to integrate a range of physiological mechanisms and bodies of empirical evidence to further our knowledge of the complex relationship between microbiota, psychological distress, and asthma (see Fig. 13.3). The association between asthma and affective disorders is complex, as many mechanisms to explain this relationship have been proposed. Microbial endocrinology has provided a new layer of perspectives that might further help our understanding of the interactions between stress and human disease. In expanding the perspective to incorporate a broader range of immune and nervous system processes as well as conceptualizations

of behavioral and experiential processes unique to psychology, a broader term such as *psychoneuromicrobiology* will become more appropriate for this emerging field. More precise modelling of trajectories of psychological development and dysregulation will be needed as the field gradually expands to studying the microbiome in humans under ecologically valid conditions.

Although in recent years research interdisciplinary teams have investigated the various potential associations that might exist between microorganisms and host cells, much remains unknown. Most of the studies that have examined the effect of stress on bacterial species have employed animal models, as have most of the studies that examined the interactions of bacterial colonization and subsequent asthma development. The extent to which these findings from animal models apply to humans is yet unknown. Another limitation of this body of literature is that most of the research to date has examined grouped asthmatics together without considering the heterogeneity of asthma as a disease entity. Therefore, most findings might only pertain to specific asthma subtypes. It is possible that the lack of attention of asthma subtypes could explain contradictory findings in the literature regarding the hygiene hypothesis. The hygiene hypothesis refers specifically to atopic asthma and therefore these findings might not extend to other asthma subtypes. It is possible that recurrent infections could lead to certain asthma subtypes while protecting against atopic asthma specifically.

Finally, studies identifying and comparing the microbial composition of the microbiome may not be sufficient to understand the interactions between microbiome and human cells. Bacterial genomes are highly variable; even if two bacterial strains belong to the same species, they may have different set of genes and therefore different interaction with human cells. This phenomenon may contribute to some of the inconsistencies and even contradictions found in the results presented by different studies (Gogarten et al. 2002).

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Chapter 14
Effects of Stress on Commensal Microbes and Immune System Activity

Tamar L. Gur and Michael T. Bailey

Abstract The body harbors a vast array of microbes that are collectively known as the microbiota. Increasing attention is being paid to the role of the gut microbiota in the health of the host. Gut microbial communities are relatively resistant to change, though alterations in homeostasis can also significantly change gut microbial community structure. An important factor that has been demonstrated to alter the composition of the gut microbiota is exposure to psychological stressors. And, evidence indicates that the commensal microbiota are involved in stressor-induced immunomodulation. This chapter will discuss the impact of psychosocial stress on immunity, and present evidence that stressor-induced alterations in the composition of gut microbial communities contributes to stressor-induced immunomodulation and neurobiological sequelae. Finally, the role of the microbiota in the perinatal time period will be explored, and an integrative hypothesis of the role of the microbiome in health and stress response will be proposed.

Keywords Microbiome • Stress • Immunomodulation • Psychosocial stress • Gut-brain axis

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14.1 Introduction

Increasing importance is being attributed to the bidirectional signaling between the gastrointestinal tract and the brain, also known as the gut-brain axis for overall health and wellbeing. The human body is colonized by a vast array of microbes, collectively called the microbiota. The great majority of these microbes reside on mucosal surfaces, including the gastrointestinal and reproductive tracts, as well as the oral cavity. It is now recognized that the gut-brain axis is vital for maintaining health and is regulated by both hormones and the immune system (Cryan and Dinan 2012). During periods of homeostasis, these microbes reside within stable communities, but exposure to stressors, including psychosocial stress, can significantly impact the structure of the intestinal microbiota. Furthermore, stressor-induced translocation of microbiota to secondary lymphoid organs may cause inflammation and activation of adaptive immunity, as well as other downstream consequences. However, much remains unknown regarding the specific mechanisms through which stress instigates changes in the microbiota, and how alterations in the microbiome influence the immune system and the central nervous system (CNS). There is already an abundance of evidence that stressor exposure leads to changes in adaptive and innate immunity, but whether these effects involve the microbiota has not been extensively studied. In this chapter, we will review the relationship between psychosocial stress, the indigenous microbiome, and the host immune system.

14.2 Psychosocial Stress, the Stress Response, and the Impact on Immunity

The ability to adapt to stressful stimuli successfully is important for every organism in order for it to survive in a changing environment. Stress is loosely defined as the process by which a stimulus (termed a stressor) disrupts internal homeostasis resulting in a physiological response (termed the stress response) that is aimed at returning the body to a state of homeostasis. While stimuli that trigger a stress response can be quite diverse (stressors can be physical, physiological, and psychological in nature), the stress response is largely conserved across different types of stressors. The stress response is a complex set of behavioral, neuronal and endocrine, responses that prepare the organism to cope with the stressor, and thus maintain internal homeostasis. Although initiation of these stress responses is typically adaptive, in that they assist the organism to survive, chronic or inappropriate activation underlies the pathophysiology of several medical and psychiatric disorders. For example, individuals exposed to stress have an increased rate of mood disorders (McLaughlin et al. 2010), coronary disease (Emeny et al. 2013) and type II diabetes (Huth et al. 2014). In addition, the functioning of the immune system is

strongly affected by the physiological stress response. Traditionally, research has been focused on the effects of stressor-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and the resultant glucocorticoid release, which can suppress immune system reactivity by suppressing the expression and activity of key transcription factors, such as NF-kB (Bellavance and Rivest 2014). Another contributor to the effect of stressor exposure on inflammation is the sympathetic nervous system (SNS), as catecholamine release (namely epinephrine from the adrenal medulla, and norepinephrine from adrenergic nerve terminals) can significantly increase or decrease immune cell activity depending on the leukocyte subset, and the adrenergic receptor that is bound. Pre-treatment with a beta-adrenergic antagonist abrogated both the anxiogenic and pro-inflammatory effects of stress (Hanke et al. 2012), demonstrating a link between SNS activation and stressor-induced immunomodulation.

While the stress response is capable of suppressing the immune response, there is now sufficient evidence indicating that the stress response can enhance or potentiate both innate and adaptive immune responses (Brydon et al. 2005; Brydon and Steptoe 2005; Steptoe et al. 2007). Social stressors in mice can enhance the reactivity of cells of both innate and adaptive immunity (Bailey et al. 2009a, b, 2010; Mays et al. 2010) e.g., the reactivity of splenic macrophages to microbial antigens is significantly increased after stressor exposure (Allen et al. 2012a, b). Even in the absence of pathogen challenge, exposure to this type of stressor increases cytokine levels, including IL-1β, TNF-α, and IL-6 in circulation (Avitsur et al. 2005; Engler et al. 2008; Bailey et al. 2011; Stark et al. 2002). The mechanisms by which stressor exposure increases immune system activity are not known, though the commensal microbiota are thought to be involved (Bailey et al. 2011; Allen et al. 2012a; Maslanik et al. 2012). Stressor exposure significantly changes the gut microbiota community structure (Bailey et al. 2006, 2011; Galley et al. 2014a) and results in the translocation of low levels of microbiota from the lumen of the intestines to the interior of the body (Bailey et al. 2006). To determine whether the microbiota could be contributing to stressor-induced immunomodulation, mice were treated with broad spectrum antibiotics to reduce the microbiota prior to stressor exposure. This antibiotic treatment prevented the stressor-induced increase in serum cytokines (Bailey et al. 2011). Likewise, the use of germfree and recolonized germfree mice (Allen et al. 2012a) has shown that microbiota are needed for stressor-induced increases in serum cytokines and enhanced innate immunity.

14.3 Overview of the Indigenous Microbiome

The commensal microbiota, which includes bacteria, viruses, fungi, and other eukaryotic species, outnumbers the cells of the human body by a factor of 10; there are approximately 10^{14} bacterial cells and 10^{13} human cells in the body (Berg 1996, 1999). While the GI tract contains 90 % of the microbiome, all external surfaces of

the body including skin, nasal and oral cavities, upper respiratory tract, and the reproductive tract are colonized as well. Molecular analysis of the intestinal microbiota using 16s ribosomal RNA now indicate that individuals can harbor as many as 1000 different bacterial species within the GI tract (Lozupone et al. 2012). These microbes should not be conceptualized as pathogenic or as opportunistic colonizers; the microbiota are symbiotic organisms with many beneficial contributions to the host, including drug metabolism and macronutrient digestion (Wikoff et al. 2009). The importance of the microbiota in the development and maintenance of the immune system is an area of increasing scrutiny and study. The immune system is comprised of innate and adaptive components, and capable of reacting to a wide range of challenges. The development of the immune system, especially adaptive immunity, is concurrent with the acquisition of the microbiota; this suggests that the microbiota serves to modify the immune system and that the immune system plays a vital role in the symbiotic nature of the relationship. Indeed, germ free (GF) mice have lower levels of serum immunoglobulins, smaller Peyer's patches, decreased cytokine production, and fewer intraepithelial lymphocytes (Shanahan 2002). When intestinal microbiota are introduced some, but not all, components of the mucosal immune system are restored (Stepankova et al. 1998; Gordon et al. 1997; Umesaki et al. 1993, 1995). In addition to metabolic and immune activity, the intestinal microbiota creates a barrier to pathogens. Members of the genus *Bifidobacterium* and *Lactobacillus* are the bacterial types most associated with colonization exclusion, as attachment of these microorganisms to intestinal cells provides a barrier to enteric pathogens (Fernandez et al. 2003; Kailasapathy and Chin 2000). These beneficial properties lend themselves to the use of *Lactobacillus* and *Bifidobacterium* as probiotics. Probiotics are defined by the World Health Organization as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). Probiotics have been studied for their usefulness in the prevention and treatment of a wide range of gastrointestinal disorders including infectious diarrhea, irritable bowel syndrome, inflammatory bowel disease, and lactose intolerance. With the emerging importance of the gut-brain axis, probiotics are increasingly being considered for their therapeutic potential for emotional disorders and mental illness (Dinan et al. 2013).

14.4 Stress-Induced Alterations in Intestinal Microbiota

While the structure of the microbial community is relatively stable, it can be disrupted by environmental, physiological, and stressful challenges. Early studies from Tannock and Savage demonstrated that moving mice into a cage lacking bedding, food, and water reduced the number of lactobacilli that could be cultured from the gastrointestinal tract (Tannock and Savage 1974). Though this suggested that the stress response following the move to new housing caused these differences, these data were confounded by food and water deprivation. Thus, following a maternal

separation stressor, infant rhesus monkeys with ad libitum access to food and water were assessed for the number of lactobacilli that could be cultured. It was demonstrated that stress significantly reduced lactobacilli levels, and the magnitude of the reduction was associated with stress-indicative behaviors. In general, monkeys who showed the greatest behavioral signs of distress also had the lowest levels of lactobacilli (Bailey and Coe 1999).

It is not yet known how stressor exposure impacts the composition of the gut microbiota, however, it is possible that these effects involve alterations to gastrointestinal physiology. The central nervous system (CNS) and the gastrointestinal (GI) tract are closely related, and stressor exposure, through glucocorticoid hormones and noradrenergic innervation, can significantly impact gastrointestinal physiology. Indeed, activation of the SNS reduces gastric acid secretion (Shichijo et al. 1993), reduces gastric emptying, slows transit in the small intestine (Nakade et al. 2005; Wang and Wu 2005), and increases motility in the colon (Nakade et al. 2007). Because GI physiology plays a large role in determining which microbes reside as part of the microbiota, as well as the levels to which those microbes can grow, it is likely that stressor-induced alterations in GI physiology impact microbiota community structure.

In addition to changes in gastrointestinal physiology, additional mechanisms may also contribute to the impact of stress on the microbiome, including direct effects of the neuroendocrine system on the microbes themselves. Gut microbiota can directly respond to hormones resulting in increased ability to adhere to mucosal surfaces (Green et al. 2004), which enhances pathogenicity. Bacterial growth rate can also be altered by exposure to stress hormones. For example, the growth of commensal and of pathogenic E. coli can be increased over 10,000-fold by adding norepinephrine to a serum-based microbial medium (Freestone et al. 2002; Lyte and Bailey 1997; Lyte 2004; Lyte and Nguyen 1997). Increased bacterial growth has also been demonstrated following stressor exposure (Bailey and Coe 1999; Pullinger et al. 2010). As stressor exposure triggers both alterations in GI physiology as well as a concomitant alteration in hormones, it logically follows that the gut microbiome is altered during stress. The growth of many types of bacteria, including both infectious and commensal organisms can be significantly impacted by neuroendocrine hormones. It is now recognized that a wide variety of neuroendocrine hormones can impact a vast array of bacteria in vitro and in vivo (Freestone et al. 2008; Lyte 2004; Bailey et al. 1999; Lyte and Bailey 1997).

The first evidence that stressor exposure impacts commensal microbes involved the use of culture-based methodology. While these studies are important, and demonstrate that stressor exposure can in fact impact gut microbes, they were limited to an assessment of a small number of commensals. The development, and widespread use, of next generation, high throughput sequencing methodologies to investigate microbial communities has significantly expanded our understanding of the impact of the stress response on commensal gut microbes. There are now multiple studies that demonstrate that stressor exposure impacts overall microbial community structure (Galley et al. 2014a; Bailey et al. 2011; O'Mahony et al. 2009).

14.5 The Functions of Microbiota During Stressor Exposure

Demonstrating alterations to gut microbial community structure following stressor exposure is a critical component of determining its importance in host physiology; understanding the biological function of altered microbiota in the host is equally essential. Immunomodulation is one potential route one potential effect that an altered bacterial population may have on the host. Stressor exposure reduces the relative abundance of bacteria in the genus *Lactobacillus*, and because the lactobacilli are known to have immunomodulatory properties (Goyal et al. 2013; Thomas et al. 2012; Mackos et al. 2013), studies from this laboratory have been focused on determining whether the lactobacilli are involved in stressor-induced immunomodulation. Mice were exposed to a prolonged restraint stressor during oral challenge with a colonic pathogen, *Citrobacter rodentium*. This pathogen induces colonic inflammation in mice, but does not possess virulence mechanisms to invade across the epithelial barrier. In mice exposed to the stressor during infectious challenge, colonic pathology was more severe and the pathogen could be cultured from systemic organs. This suggests that the pathogen was able to cross a disrupted intestinal barrier in the mice exposed to stress, but not in mice left undisturbed during pathogen challenge. This systemic pathogen translocation was also associated with increases in circulating cytokines, such as IL-6, and an increase in anxiety-like behavior. Of note, pathogen translocation to the spleen, as well as circulating IL-6 levels, was significantly reduced in stressor-exposed mice fed *Lactobacillus reuteri* (Fig. 14.1). This probiotic also prevented the development of anxiety-like behavior in the mice exposed to the prolonged restraint stressor during oral challenge with *C. rodentium* (Mackos et al. 2013). These data suggested that exposure to a prolonged stressor increased susceptibility to *C. rodentium*, and that probiotic treatment alleviated the effect of stress. It is likely that this effect is dependent upon alterations in the commensal microbiota. Studies by this laboratory, as well as others, have shown that stressor exposure reduces commensal lactobacilli (Galley et al. 2014a). As administration of lactobacilli prevented some systemic manifestations, it suggests that stress-induced reduction in lactobacillus is responsible for some of the systemic effects of stress on *C. rodentium*.

14.6 Perinatal Stressor-Induced Alterations to Microbiome Development

Scientific interest in the connection between stress and the human microbiome, and its impact the developing fetus is increasing, but still in an early stage. The microbiota may have exaggerated and prolonged effects when perturbed during gestation or early in infancy. Pregnant rhesus monkeys exposed to auditory stressor during their 24 week gestation demonstrated an increased level of cortisol, and their offspring demonstrated significantly lower levels of lactobacilli. In addition, though not intentionally exposed to pathogen, the offspring of stressed mothers

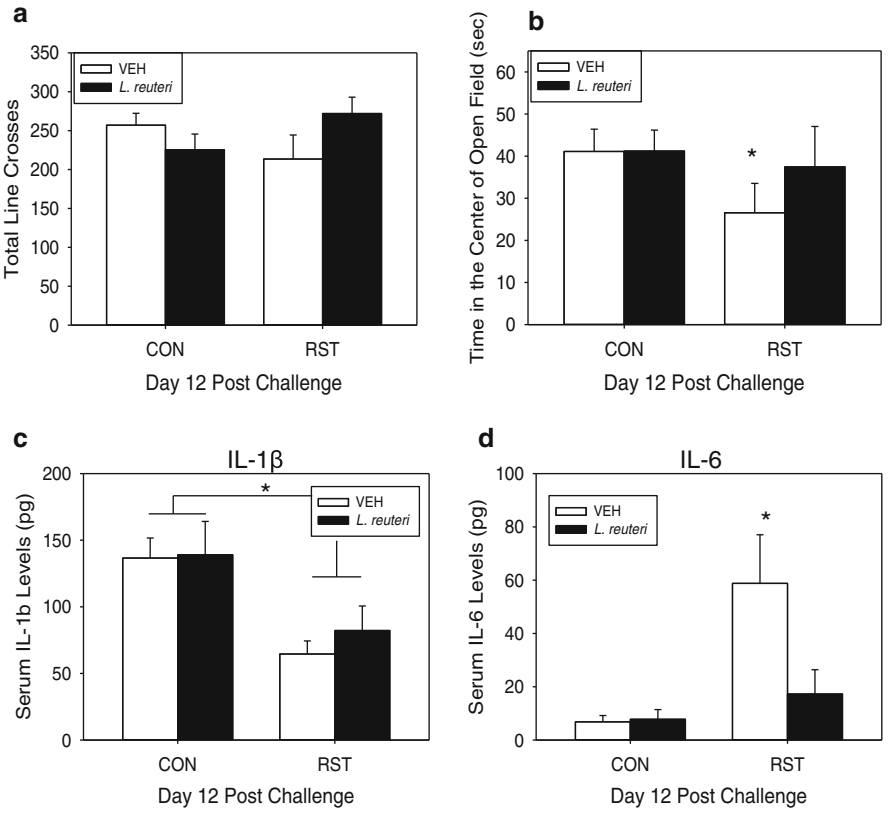

Fig. 14.1 From: Probiotic Lactobacillus reuteri Attenuates the Stressor-Enhanced Severity of Citrobacter rodentium Infection. Probiotic intervention reduces stressor-enhanced anxiety-like behavior and significantly reduces circulating IL-6 at the peak of infection. Mice were restrained for 1 day and then orally challenged with *C. rodentium*. Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either VEH or *L. reuteri*, beginning 1 day prior to restraint. Prior to sacrifice, mice were tested by the open field test and recorded for 5 min. Locomotion was quantified by the total number of lines crossed and the amount of time spent in the center of the open field. (**a**) There were no changes in the total number of lines crossed in either stressor-exposed group. Sample sizes were as follows: $n=12$ (CON/VEH), $n=12$ (CON/L. reuteri), $n=6$ (RST/VEH), and $n=4$ (RST/L. reuteri). (**b**) There was an increase in anxiety-like behavior, as evidenced by a reduction in the time spent in the center of the open field, in VEH-treated, stressor-exposed mice, which was restored with L. reuteri treatment. $*P<0.05$ between CON/VEH and RST/VEH groups as assessed by Student's *t* test with the Bonferroni correction factor as a *post hoc* test. Sample sizes were as follows: $n=18$ (CON/VEH), $n=18$ (CON/L. reuteri), $n=9$ (RST/VEH), and $n=9$ (RST/L. reuteri). Cytokine levels were determined by a Bio-Plex assay. (**c**) Circulating IL-1β was reduced by stressor exposure on day 12 postchallenge, and L. reuteri failed to modulate this reduction. *ANOVA main effect for group (CON versus RST; $P=0.01$). Sample sizes were as follows: $n=13$ (CON/VEH), $n=11$ (CON/L. reuteri), $n=7$ (RST/VEH), and $n=7$ (RST/L. reuteri). (**d**) Circulating IL-6, however, was significantly increased by stressor exposure in VEH-treated mice on day 12 postchallenge. Probiotic intervention with L. reuteri reduced stressor-enhanced circulating IL-6 levels. $*P<0.001$ between CON/VEH and RST/VEH groups. Student's *t* test with the Bonferroni correction factor was used as a *post hoc* test. Sample sizes were as follows: $n=14$ (CON/VEH), $n=13$ (CON/L. reuteri), $n=7$ (RST/VEH), and $n=6$ (RST/L. reuteri). Figure originally published in: Amy R. Mackos, et al. Infect Immun. 2013 September;81(9):3253–3263

demonstrated decreased resistance to *Shigella flexneri*, an endemic pathogen within the colony (Bailey et al. 2004).

While it is recognized that prenatal gestation may impact the development of the microbiota, leaving the offspring susceptible to infection, the effects of gestational stressors may actually be manifest even prior to infant development. Stress is a well-known risk factor that affects many factors that have been associated with preterm birth, including the immune response, inflammation, and the HPA axis (Shapiro et al. 2013). These factors may be significantly impacted by the commensal microbiota (Gur et al. 2015). Microbial invasion of the amniotic cavity and subsequent host inflammatory response is a prime suspect in the causation of PTB (DiGiulio 2012; Goldenberg et al. 2000; Goncalves et al. 2002). Microbes are thought to invade both hematogenously and from the lower gut or genital tract (DiGiulio 2012). While genital pathogens and their capacity to invade the amnion and induce PTB have been well studied, the source of hematogenous microbes and their ability to invade the amniotic cavity remains unclear. Oral microbes have been implicated, based largely on a study that demonstrated that the placenta harbors a commensal microbiome community structure that is reflective of oral microbial communities (Aagaard et al. 2014). While it is known that stressor exposure can change the composition of the gut microbiota, and the ability of microbes to translocate from the lumen of the intestines to the interior of the body (Bailey et al. 2006, 2010; Galley et al. 2014b), the relationship between the various indigenous microbial communities and the intrauterine environment is not well understood.

Animal studies support the idea that stress-induced alterations in neonatal life and gut microbiome can have substantial impact on long-lasting health. Rat offspring deprived of their mother demonstrated increased permeability of colonic mucosa and increased bacterial adherence to colonic tissue and spleen translocation. The HPA axis likely mediated this effect, as the negative sequelae were prevented by injecting rat pups with a corticotropin-releasing hormone receptor antagonist during maternal separation (Gareau et al. 2006). A swine model for perinatal disturbance was adopted with oral antibiotics to sows. Short- and long-term changes were seen in paracellular permeability (Boudry et al. 2011). Together, these studies suggest that altering either the maternal or perinatal microbiota has long lasting implications on the gut of the offspring, as well as immunological repercussions. Mechanisms for this change are still being evaluated, but one potential route is that perinatal changes in bacterial colonization alter gastrointestinal heat shock protein expression with permanent implications on health (Lalles 2012).

14.7 Towards an Integrative Understanding of Stress, Microbiota, and Immunity

The significance of stressor-induced changes in the commensal microbiota is only beginning to be understood. The role of the microbiome in human disease has expanded from the intestines to other organ systems; the biological mechanisms and

underpinnings through which the microbiome exerts its effects are only beginning to be understood. Microbiota-induced alterations in the host inflammatory response are almost certainly important for stressor-induced immunopathology. The ability of stressors to impact host inflammatory responses is well documented in both rodents and humans, even in the absence of overt infection (Brydon et al. 2006; Steptoe et al. 2007; Coussons-Read et al. 2007; Bierhaus et al. 2003; Avitsur et al. 2002; Bailey et al. 2007; Engler et al. 2008; Stark et al. 2001). The questions of why and how the immune system is activated during a stress response, even in the absence of antigen challenge, have long been postulated. Given what we now know regarding the effect of stress on the microbiome, it is hypothesized that stressor-induced changes in the commensal microbiota activates the immune system, resulting in stress-induced elevations in cytokines. Demonstrating how these alterations occur, and determining how to optimize pre- and probiotic interventions that can shift an individual from disease to health is the next challenge for this field to tackle.

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Chapter 15
Microbiome to Brain: Unravelling the Multidirectional Axes of Communication

Sahar El Aidy, Roman Stilling, Timothy G. Dinan, and John F. Cryan

Abstract The gut microbiome plays a crucial role in host physiology. Disruption of its community structure and function can have wide-ranging effects making it critical to understand exactly how the interactive dialogue between the host and its microbiota is regulated to maintain homeostasis. An array of multidirectional signalling molecules is clearly involved in the host-microbiome communication. This interactive signalling not only impacts the gastrointestinal tract, where the majority of microbiota resides, but also extends to affect other host systems including the brain and liver as well as the microbiome itself. Understanding the mechanistic principles of this inter-kingdom signalling is fundamental to unravelling how our supraorganism function to maintain wellbeing, subsequently opening up new avenues for microbiome manipulation to favour desirable mental health outcome.

Keywords Microbiota • Gut-brain axis • Immune system • Metabolites • Epigenetics

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15.1 Introduction

In the last decade, studies on the human microbiome have attracted tremendous attention in an effort to understand the relationships between the host systems and aspects of the host microbiome that can point to novel mechanisms underlying host regulation of the microbiome. The human intestine harbours the majority of the vast and diverse body's microbes that has co-evolved with its host and is essential for human health (Gordon 2012). The two major research consortia, the American Human Microbiome Project (http://commonfund.nih.gov/hmp) and the European MetaHIT project (http://www.metahit.eu) have both unravelled a great deal of knowledge related to intestinal bacterial communities in humans (Human Microbiome Project Consortium 2012; Arumugam et al. 2011; Qin et al. 2010; Nielsen et al. 2014). Both take advantage of next generation sequencing to reveal the large variability in microbiota profiles between individuals.

The gut microbiota has coevolved with the human host to perform a number of beneficial functions by protecting against pathogens, ferment indigestible nutrients, produce micronutrients, and metabolize drugs and harmful toxins (Tremaroli and Backhed 2012; Nicholson et al. 2012). However, it has become increasingly appreciated that the role of the gut microbiota extends beyond the gastrointestinal (GI) tract to help balance host vital functions and participate in maintenance of health and wellbeing (Lyte and Cryan 2014; Blaser 2014). The human microbiome is also thought to play a key role in evolution; the concept of hologenome theory, which proposes that the holobiont is "a long-term physical association between different living organisms" (Lynn 1991). It has furthermore been suggested that the holobiont is a unit that can undergo natural selection (Brucker and Bordenstein 2013). The host selects for certain microbiota that are capable of establishing a symbiotic relation with the host environment as well as with other microbial members of the gut ecosystem (Arumugam et al. 2011). In fact, the intimate host-microbiome interactions postulate that any changes within the host are associated with changes in its microbial genome (Gilbert et al. 2010). In turn, the unit of genome selection becomes no longer the host's but it's hologenome, i.e. the genome of both the host and microbiome (Sharon et al. 2010).

15.2 The Microbiome: Our Other Selves

In recent years, the complex and dynamic intestinal microbiota has attracted great interest, ranging from being simple fermenters of food to having profound effects on human physiology, nutrition, immunity and even mood and behaviour (Blaser 2014; De Vos and De Vos 2012; Cryan and Dinan 2012; Surana and Kasper 2014). Consequently, disruption in or alterations of the intimate cross-talk between the microbes and human cells may be a significant factor in many diseases such as obesity, type 2 diabetes, gastrointestinal disorders, stress and major depression (Nicholson et al. 2012; Cryan and Dinan 2012). The human gastrointestinal (GI)

tract is estimated to harbour around a thousand billion bacteria, which corresponds to 10–100 times more than the number of the human body cells (De Vos and Nieuwdorp 2013). The diversity of the gut microbes is substantial, estimated to be around a 1000 different bacterial species, despite the relatively low phylum-level diversity, which is dominated by the phyla of the Bacteroidetes, Firmicutes with Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia phyla present in lower abundance (Eckburg et al. 2005; Qin et al. 2010). Moreover, the community structure of these different species is highly dynamic and can vary markedly between individuals, from 10- to 1000-fold (Qin et al. 2010). Yet it is obscure whether this variation is driven by the differences between individuals in genetic backgrounds, or by different diets and other environmental influences.

Humans live in constant association with microbes that are present on surfaces and in cavities of the human body, and even within the cells, thereby a detailed understanding of human biology requires knowledge of both human genome as well as human microbial metagenome (Baltimore et al. 2008). Understanding our other genome will open new avenues to shape the gut microbiome in a way that is therapeutically beneficial for individuals whose gut microbiome is unbalanced. Unlike the mammalian core genome, which is relatively constant, microbial metagenomes are surprisingly plastic (Patterson and Turnbaugh 2014).

Being anaerobes and inhabiting a milieu that is difficult to characterize and reproduce *in vitro*, the majority of the gut microbiota cannot be cultured in the laboratory. Consequently, it was impossible to assign genes to un-cultivable bacteria because the metagenomic analysis was based on comparing the genes detected in a sample with those of genes from bacteria that could be cultivated in a laboratory, which represent only around 15 % of the gut bacteria (Wood 2011). In the past few years, advances in rapid and inexpensive sequencing technology have made it possible to sequence and assemble the complete genomes of 238 gut bacteria, the majority of which were previously unknown (Qin et al. 2010). In another study, data from nearly 400 human gut microbiome samples shed light on microbial communities in humans by clustering millions of genes into a few thousand co-abundance groups of genes (Nielsen et al. 2014). Approximately 10 % of these groups of genes corresponded to bacterial species referred to as metagenomic species, 85 % of which represented unknown bacteria species. The other minor groups corresponded to bacterial viruses, along with bacteriophages, plasmids or CRISPR sequences, which protect bacteria from viral attack.

There are growing attempts to characterize microbial communities in various sites of the human body, including the GI tract, into ‘enterotypes’, that similar to blood groups identify the individual as host to a specific composition of intestinal bacteria (Siezen and Kleerebezem 2011). Indeed, the METAHIT project led to the suggestion of clustering humans into three separate enterotypes based on the composition of their gut bacterial communities but independent of geography, short-term diet or skin colour (Arumugam et al. 2011). The identified enterotypes were characterised by the predominant bacterial population: Bacteroides, Prevotella and Ruminococcus. However, linking specific dietary habits to microbial enterotypes has been a heuristically attractive discovery (Wu et al. 2011), which is in line with

finding highlights the significance of the diet in determining the community structure of the health-shaping microbiome. Although the concept of classifying the intestinal microbiome into "enterotypes" provides an attractive way to understand microbial variation in health and disease, it is somewhat simplistic, has been challenged conceptually and there is emerging evidence against discrete types of microbiome urging the need for appropriate statistical description of the microbiome (Knights et al. 2014).

15.2.1 We Are What Our Microbiomes Eat

Emerging studies demonstrate that the make-up of the microbiome is related to the expanding repertoire of diseases associated with modern living (Patterson and Turnbaugh 2014). For example, some individuals develop metabolic disorders and are more susceptible to the 'obesogenic' environment than others. The reports suggest an important inherited component, as concluded from several twin- family- and adoption studies, yet the proportion of explained genetic variance of body mass index remains low. Nonetheless, emerging evidence suggests that variation in the microbiome may have an even greater role than human genome variation in the pathogenesis of obesity. Turnbaugh and colleagues showed that the differences observed in obese and lean mouse microbiotas were conserved in obese people and could be influenced by caloric restriction (Turnbaugh et al. 2006). However, other studies could not directly confirm these initial findings and suggested that obesity may be associated with more subtle changes in the microbiota composition (Schwiertz et al. 2010; Zhang et al. 2009; Duncan et al. 2009). In addition, some studies have related the changes observed in the microbial composition of obese patients to high fat, high-energy diet (Daniel et al. 2014; Cani 2013). Dietary perturbations, for example, can exert strong effects on the gut microbiota in few days-time as reported in a study on ten human individuals, who were fed a vegan or animal-based diet and their gut microbes were rapidly and reproducibly altered in response (David et al. 2014).

Despite the accumulating data from laboratory animals, the question remains unanswered is whether the changes in the microbial composition are a direct effect from altered nutrient availability in the GI tract or are consequences of the effect of altered diets on host physiology that are consistent across genotypes. A human study examined the metagenome in the stool of nearly 300 lean and obese Danish volunteers, as well as markers of metabolic health (Le Chatelier et al. 2013). The study showed that having a relatively low genetic diversity in an individual's microbiome correlated with higher inflammation, greater insulin resistance and other warning signs of metabolic diseases. Obese participants who had low diversity also gained significantly more weight over the course of 9 years. Another study conducted in obese volunteers, who were put on a low-caloric diet, demonstrated an increase in the genetic diversity of the microbiome and improved metabolic markers in those who

had low diversity in their microbiomes at the start of the intervention. In contrast, people who already had high diversity didn't show much improvement compared to the former group, suggesting that a low-diversity microbiome, though linked to worse metabolic health, can be subject to dietary intervention (Cotillard et al. 2013).

Another important aspect is the central regulation of obesity and food intake, in general (Morton et al. 2006). Gut microbiota appear to influence the peripheral control of food intake and obesity but whether it also has an impact on the central regulation remains obscure. Such studies are now warranted, especially given the rapidly expanding obesity epidemic. Notably, a common side effect of centrally acting psychotropic drugs is obesity, and we have shown that the gut microbiota mediate at least in part these effects based on the finding that gut microbiota composition was altered following treatment with olanzapine in rats (Davey et al. 2013).

15.3 Epigenetics and the Neonatal Microbiome: Journey Not Destination Matters

The neonatal microbiota colonizes at birth, although evidence is beginning to emerge that the in utero environment may not be sterile as initially believed (Funkhouser and Bordenstein 2013) since bacteria such as *Escherichia coli*, *Enterococcus fecalis*, and *Staphylococcus epidermidis* were detected in the meconium of healthy neonates (Jimenez et al. 2008). The microbial community of the new-born gut is initially undifferentiated throughout the GI tract, more dynamic in its composition and less stable over time (Nylund et al. 2014). Particularly, in the first year of life, the GI tract progresses from sterility to enormously dense colonization, characterized by a mixture of microbes that is relatively stable and largely similar to that found in the adult intestine (Palmer et al. 2007; Nylund et al. 2014). A range of factors, including pre- and postnatal as well as birth factors, is involved in shaping the composition of the gut microbiota (Mueller et al. 2015; Borre et al. 2014). The method of delivery, breast-feeding, weaning, and antibiotic treatment are among the factors that influence the microbial community during this stage of development (Penders et al. 2006; Lemon et al. 2012). For example, infants born via caesarean section (C-section) show markedly different bacteria not only in their gut but also on their skin, noses, mouths and rectums in comparison to babies born vaginally. Infants born vaginally were colonized predominantly by Lactobacillus *spp*, microbiota that helps in the digestion of milk oligosaccharides (Johnson and Versalovic 2012). The C-section infants, however, were colonized by a mixture of potentially pathogenic bacteria that are usually found on the skin and in hospitals, such as Staphylococcus and Acinetobacter (Johnson and Versalovic 2012; Mueller et al. 2015). Although these differences in the composition of the microbiota are temporary, previous studies suggest that C-section born babies are more likely to develop allergies, asthma and other immune and neuronal system–related disorders than are babies born vaginally (Mueller et al. 2015).

15.3.1 Microbe-Immune-Brain Interfaces: Sites for Epigenetic Regulation

While the term epigenetics is now often used in a rather broad sense, within scientific disciplines it may have slightly different nuances. In the most general terms, epigenetic mechanisms are used to describe the regulation of gene expression in a programmed manner, as for example during development. In neurosciences, the term epigenetics is often used to accommodate the fact that this molecular epigenetic machinery is intimately involved in the dynamic regulation of neuronal gene expression, including plastic changes in nuclear architecture, chromatin structure and remodelling. Three main molecular mechanisms constitute the molecular epigenetic machinery: Histone modifications, such as acetylation, methylation or phosphorylation, DNA modifications, such as CpG-methylation, and regulatory RNAs. Thus, epigenetic regulators control the amount of expression of a gene by integrating external and intracellular signalling cascades transcriptional or translational level.

Dynamically regulating neuronal gene expression, all of these processes have been demonstrated to be necessary for brain development and function throughout life (for recent reviews see Fischer 2014; Woldemichael et al. 2014). The first observations have been made studying the dynamics of transcription during long-term memory formation, which critically depends on *de novo* gene expression (Da Silva et al. 2008). Aiding in this process, a key role learning-induced gene regulation seems to be histone acetylation, which is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). These conserved protein families are well understood and currently under investigation as promising targets in neurodegenerative diseases and cognitive decline (Stilling et al. 2014b).

In addition to histone and DNA modifications, also non-coding RNAs (ncRNAs) and RNA modifications are now well established as modulators in development, normal brain function and neuropsychiatric disease (Qureshi and Mehler 2012; Barry and Mattick 2012). Most prominently these are small RNAs, incl. miRNAs (O'Connor et al. 2012; Saab and Mansuy 2014) and piRNAs (Landry et al. 2013), and lncRNAs (Schaukowitch and Kim 2014; Ng et al. 2013). More recently also recoding of the genomic information by RNA editing, neuronal activity-dependent alternative splicing (Schor et al. 2009) as well as RNA methylation (Meyer et al. 2012) have recently been added to the list of RNA-dependent mechanisms that alter gene expression or the function of the encoded protein in response to external stimuli. Interestingly most of these processes are enriched in the brain compared to other tissues. Importantly, many of these RNA-based processes are not only interacting with each other (Barry and Mattick 2012) but are also intimately linked to the other epigenetic processes. There is now a growing appreciation of the role of epigenetic mechanisms in shaping brain and behaviour and mediating at least some of the effects of the microbiota on host regulatory programs, even though the underlying molecular mechanisms leading to these behavioural and biochemical alterations are not well understood.

15.3.2 They Come to Stay: Early-Life Events Affect Microbiota and Brain Development

Early-life establishment of the immune system is heavily influenced by the colonization with diverse commensals, which offers a plethora of antigens that are crucial for appropriate maturation of the immune system as evidenced by reports from germ free (GF) animals exhibiting exhibit severely immature immune function (Cebra 1999; Hooper et al. 2012; Cahenzli et al. 2013). Moreover, it is well-documented that maturation and maintenance of the immune system also depend on epigenetic modifications that govern the expression of immune-related genes and transcriptional profiles of immune cells (Weng et al. 2012; Stender and Glass 2013). As a recent proof-of-concept study, Kumar et al. reported that changes in DNA methylation in human blood samples were associated with the dominant bacterial phylum prevalent in faecal samples (Kumar et al. 2014). Interestingly, multiple authors suggested epigenetic mechanisms to mediate interactions between the host and its associated microbiota by, even though largely with respect to pathogenic or parasitic microbes (Gomez-Diaz et al. 2012; Minarovits 2009; Paschos and Allday 2010; Al Akeel 2013; Silmon De Monerri and Kim 2014; Kim 2014). More recently, these concepts were extended/amended to include also non-pathogenic microbiota (Stilling et al. 2014a, b; Shenderov and Midtvedt 2014), which may be an important target of future research in the context of brain and behaviour. Recently, the intestinal microbiota has been also shown to modulate homeostasis and inflammatory response of the intestinal epithelium in an HDAC3-dependent manner (Alenghat et al. 2013), thereby establishing a direct connection between microbiota and epigenetic gene regulation.

It is now well established that the effects of early-life events on adult behaviour are mediated by epigenetic mechanisms (Kundakovic and Champagne 2015). Notably, there is also increasing evidence that there is mutual interaction between the microbiota and the effects of early-life events and potential convergences between epigenetic mechanisms and host-microbiota dialogue are emerging from studies exploring the impact of early-life events on both, microbial composition and brain function. Using maternal separation as an early-life stressor in rats it was demonstrated that postnatal adversity, next to its effects on anxiety- and depressive-like behaviours, changes the composition the intestinal microbiota and induces visceral hypersensitivity later in life (O'Mahony et al. 2009, 2014; Hyland et al. 2015). Thus, there is a strong indication for an interaction between early development of brain and microbiota and that this interaction epigenetically programs adult behaviour and the response to stress (Jasarevic et al. 2015).

Early colonization is also linked to activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn impacts the enteric nervous system (ENS) that innervates the GI tract (Borre et al. 2014; Walker 2013). The beneficial effects of the early colonizing microbiota in developing immune and nervous systems, extracting nutrients from food and keeping harmful microbes at bay, presumably via the process of colonization resistance (El Aidy et al. 2013c), support the concept of the presence of a critical window of development during the early life that allows for a

full-scale establishment of an adequate microbiota-accommodating homeostasis. This notion coincides the microbiota-deficiency hypothesis, which postulates that colonization with a "healthy" microbiota during the vulnerable developmental period exerts effects that may decrease susceptibility to diseases, whereas its absence or dysbiosis, as in antibiotic treatment in childhood, may have reverse effects (Rook 2013).

Throughout adulthood, the gut microbiota seems to be more stable although adolescents have been reported to harbour a higher abundance of Bifidobacteria and Clostridia in comparison to adults (Agans et al. 2011). As a final age-related microbial shift, changes in the function and composition of the gut microbiota occurs during old age, which is associated with changes in physiological functions, including a decline in the immune system (Claesson et al. 2012). A shift in the ratio of Bacteroidetes to Firmicutes, distinct decrease in Bifidobacteria, and an overall increase in the total number of facultative anaerobes were observed in aged people (Mariat et al. 2009). Age-related changes in the microbiome is also mirrored in the host excretion profiles (urine and faeces) of bacterial-dependent metabolites (Nicholson et al. 2012) highlighting the significance of host-microbe co-metabolism in governing their intimate interactions.

15.4 A Microbiome Industrial Age: The Bodies Bacterial Bioactive Factories

The chemical dialogue between the microbiome and host cells involves small molecule signalling (metabolites, peptides and proteins) and varies along different regions of the GI tract, which inhabit different members of the commensals (Gordon 2012; El Aidy et al. 2013a; Nicholson et al. 2012). These signalling molecules play a key role in shuttling information between the host cells and its microbiota. In fact, about one third of the metabolites circulating in the blood depends on the microbiota for their synthesis (Bourzac 2014). Even cerebral metabolites are influenced by our microbiome, signifying the impact of intestinal microbiota on brain health and disease (Matsumoto et al. 2013). During the metabolism of food and xenobiotics, the gut microbiota can carry out a wide range of biotransformation reactions, including those that are not present in the mammalian host (Van Duynhoven et al. 2011). Indeed, several dietary components have limited bioavailability in their intact form; thereby require the gut microbiota to convert them into metabolites with beneficial health effects that can undergo further metabolism upon entering systemic circulation (Fig. 15.1). Dietary nutrients, such as polyphenols, act as prebiotics that affect both the composition and metabolism of the intestinal microbiome (Bolca et al. 2013). Polyphenols have been shown to increase the abundance of beneficial microbes such as Bacteroides, Lactobacillus and Bifidobacterium (Jin et al. 2012; Tzounis et al. 2011) but also inhibit the growth of potentially pathogenic bacteria. For example, Catechin was found to significantly inhibit proliferation of the pathogenic Clostridial species (Tzounis et al. 2008). Through their effect on the

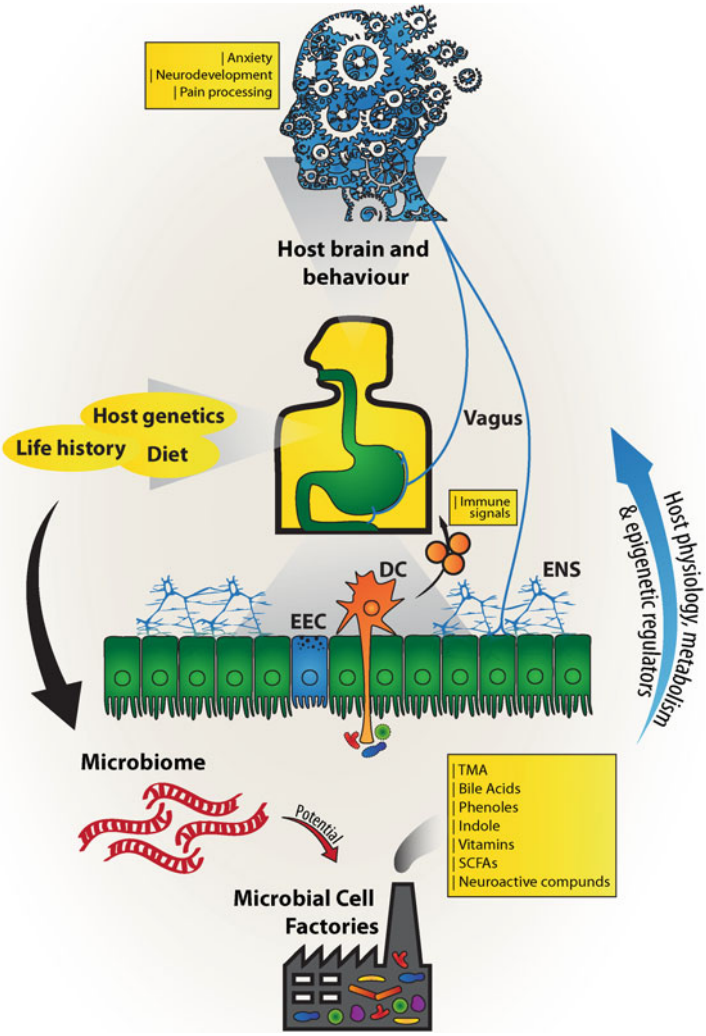

Fig. 15.1 *The microbiome industrial age*. Host genetics, life style and available nutrients (diet) are the main determinants of the composition and diversity of the intestinal microbiome. This lays the basis for production of microbial metabolites that act as effector molecules, yielding substrates for downstream host metabolic reactions and regulating host physiology. This, at least in part, is orchestrated by epigenetic mechanisms. In addition, the host immune system is stimulated by intestinal microbes and immune signals are relayed to other organs, including the brain. Together with direct nervous connections, these mechanisms influence brain functions, including anxiety, neurodevelopment, and pain perception. Therefore, the microbiome is an important part of a feedback loop, mediating of gene-environment interactions. Abbreviations: *EEC* enteroendocrine cell, *DC* dendritic cell, *ENS* enteric nervous system

microbiome, polyphenols can affect not only the GI tract but also other organs including the brain. Several epidemiological studies suggest that polyphenols may beneficially affect human brain function and may improve memory and cognition, presumably via free radical scavenging, or the modulation of enzyme activities (Schaffer and Halliwell 2012).

Nonetheless, only a handful of bioactive microbiota-dependent metabolites have been identified to date, with very few known to be involved in maintaining the host-microbial homeostasis (Nicholson et al. 2012). Unravelling the complex microbiota-metabolic processes and products is coming to grips with the difficulty in culturing the majority of the intestinal microbiota under standard laboratory conditions. The fact that metabolites produced by one bacterium can be utilized or modified by others adds to the complexity of the identification of microbiota-produced metabolites. Moreover, the classification of the identified metabolites as microbiota- or host-derived is another challenge since the majority of metabolites are shared between pro and eukaryotes (Peregrin-Alvarez et al. 2009). Linking the intestinal microbiota composition to functionality requires bottom-up as well as top-down approaches with combined application of *in vitro*, humanized mouse models and human intervention trials (Van Duynhoven et al. 2011). In a recent study, a metabolomics strategy was developed to facilitate the characterization of microbiota-dependent metabolites and describe which molecules are formed from which bacterial biotransformation reactions (Sridharan et al. 2014). Among the identified metabolic reactions, amino acid metabolism represented the largest group that require the microbiota for their synthesis and metabolism, as illustrated by their low abundance or absence in the caecum of GF mice. The findings of this study are compatible with previous reports plasma and urine metabolites in GF and antibiotic-treated rodents (Wikoff et al. 2009; Zheng et al. 2011).

15.4.1 Microbiota-Dependent Metabolites

TRIMETHYLAMINE (TMA) is the product of microbiota-dependent choline (essential dietary nutrient) metabolism (Dumas et al. 2006). TMA is identified only in the urine samples of ex-GF mice indicating it is exclusively produced by the gut microbiome (El Aidy et al. 2013a; Claus et al. 2008). A potential link between the intestinal microbiota, dietary choline, and cardiovascular disease risk has been suggested, where increased metabolites of the dietary lipid phosphatidylcholine have been observed in the blood of patients suffering from myocardial infarction or stroke compared to that of normal individuals (Wang et al. 2011). TMO is also associated with bad breath as seen in patients with the genetic disease trimethylaminuria that is associated with a mutation of the liver enzyme flavin-containing monooxygenase 3 (FMO), which oxides TMO into trimethylamine-N-oxide (TMAO) (Mitchell and Smith 2001). Recently, TMO was shown to be involved in species-specific social communication, via its olfactory receptor, trace amine-associated receptor 5 (TAAR5) (Li et al. 2013). Unlike in humans, TMO is detected in mouse

urine in much higher levels, where it serves as a strong attractive odour source to ensure that mating and other social behaviours are properly directed in a concentration-dependent manner. Indeed, depletion of FMO or depletion of the TAAR5 receptor was linked with decreased odour attraction in mice suggesting an impact of TMO on behaviour and brain function.

BILE ACIDS integrated metabolism in mammals represent an intriguing example of the inter-kingdom signalling between the host and microbiota. Bile acids function by facilitating the metabolism of dietary fat and the absorption of fat-soluble vitamins and cholesterol. Biotransformation of about 5–10 % of bile acids occurs through degradation by the major groups of intestinal bacteria, including Bacteroides, Eubacterium, Lactobacillus, Escherichia and Clostridium via bile salt hydrolase enzymes (Ridlon et al. 2006). Reports from GF and antibiotic treated rodents described the crucial role of the intestinal microbiota in the metabolism of bile acids. GF mice have higher levels of phospholipids in their liver and higher levels of bile acids in gut tissue, indicating that the gut microbiota is a key regulator of bile acid metabolism (Swann et al. 2011). Altered expression in genes associated with cholesterol, steroid, and bile acid synthesis as well as altered conjugated bile acid was characteristic in multiple body compartments of these animals.

Bacterial degradation of bile acids involves the deconjugation of taurine- and glycine-conjugated bile acids to unconjugated free bile acids, which in turn undergo reabsorption, mainly by bile acid transporters in the ileal epithelium but also by passive absorption throughout the intestine (Dawson et al. 2009). Through their action on bile acid metabolism, the intestinal bacteria can indirectly affect several pathways involved in energy and lipid metabolism, bile acid synthesis and transport, lipid and carbohydrate metabolism, and even the regulation of intestinal innate immunity. This action occurs via the ligation of bile salts with one of the nuclear hormone receptors; the farnesoid X receptor (Vavassori et al. 2009).

Impaired bile acid metabolism has been linked with microbial dysbiosis (Duboc et al. 2013), which in turn alters the capacity of the gut community to modify bile acids (Ogilvie and Jones 2012) resulting in impairment of the enterohepatic flow, which is required for regulation of bacterial populations and growth rates to achieve the normal balance of bacteria throughout the GI tract (Ogilvie and Jones 2012). Impaired enterohepatic flow leads also to digestion and absorption of fat-soluble nutrients and is associated with impairment in bile-acid amino transferase (BAAT), which catalyses the final reaction in the formation of the primary conjugated bile acids. Intriguingly, in a case-study of an old female who had a genetic deficiency in BAAT, showed unique pattern of elevated levels of taurine and glycine bile acids, CNS dysfunction and intestinal dysbiosis with elevated levels of *Fusobacteria*. Correction of microbial population and improved CNS functions were rapidly produced upon application of BAAT replacement therapy (Lord et al. 2014).

Bile acids-related microbial dysbiosis has also been linked with hepatic encephalopathy (Bajaj et al. 2013). Hepatic encephalopathy represents an interface for the microbiota signalling to the gut-liver-brain axis. The successful treatment of hepatic encephalopathy with antibiotics suggests the involvement of microbial dysbiosis in the aetiology of the disease. Indeed, microbiota changes in hepatic encephalopathy

have been associated with impaired cognition, endotoxemia, and inflammation, where reduced abundance of the taxa Lachnospiraceae, Ruminococcaceae, and Clostridiales XIV and increased Enterobacteriaceae and Streptococcaceae were reported (Bajaj et al. 2012). This dysbiosis is related to a reduction in hepatic bile acid synthesis, which in turn leads to more release into the intestine, and subsequent systemic inflammation. Dysbiosis in hepatic encephalopathy results also in an increase in gut-derived products such as ammonia, endotoxin, inflammatory cytokines, and bacterial DNA into the systemic circulation via the mal functioning liver (Zapater et al. 2008). Subsequently brain dysfunction including cognitive impairment and neuro-inflammation has been associated with HE (Bajaj 2014).

In summary, bile acids represent an intriguing example of co-metabolism between the gut microbiota and host and suggest that modulation of bile acid metabolism activity in the microbiota may be an effective target in the treatment of obesity and metabolic syndrome (Joyce et al. 2014).

PHENOL and phenolic derivatives are produced by the intestinal microbiota, in particular, Clostridium, Bifidobacterium, *Bacteroides fragilis* and *Escherichia coli*, from tyrosine (Bone et al. 1976). Around 50–100 mg of volatile phenols are excreted in humans per day, mainly in the form of glucuronide and sulphate conjugates of phenol or 4-cresol (Nicholson et al. 2012). Altered levels of volatile phenols in human urine have been linked to a large array of physiological and pathological conditions, including weight loss and inflammatory bowel disease (IBD). 4-Cresol produced by Clostridia was detected at significantly higher concentrations in the urine samples of children with autism spectrum disorders (ASD) and in schizophrenia and treatment with antibiotics against Clostridia species improved the autistic symptoms (Zheng et al. 2011). The underlying mechanism by which phenolic compounds produced by Clostridia contribute to the markedly altered behaviour in autism and other neuropsychiatric diseases is proposed to involve the inhibition of the conversion of dopamine to norepinephrine (Shaw 2010). Elevated levels of dopamine not only cause abnormal behaviour but also in severe brain damage.

Notably, 4-cresol (4-methylphenylsulfate) shares structural similarity with another microbial-dependent metabolite: 4-ethylphenylsulfate (4EPS) (Hsiao et al. 2013). Like, 4-cresol, 4EPS was found to induce ASD related behavioural abnormalities when injected in naïve mice. Moreover, 4EPS was dramatically elevated in serum levels of offspring of maternal immune activation (MIA), a mouse model which exhibits features of ASD. 4EPS is proposed to be produced by Lachnospiraceae family of Clostridia and ingestion of *Bacteroides fragilis* was elegantly shown to restore the serum levels of 4EPS to normal.

INDOLE is exclusively produced by the intestinal microbiota, which converts tryptophan into indole, pyruvate and ammonia by the bacterial tryptophanase enzyme (Lee and Lee 2010). Indole regulates gut immune cells and is proposed as a potential treatment of IBD via its immunomodulatory and anti-inflammatory effects on intestinal epithelial cells, which are central regulators of gut homeostasis (Bansal et al. 2010). Indole can be further modified into indole-2-acetic acid (IAA) and the neuroprotective molecule; indole-3-propionic acid (IPA). Incubation of human large intestinal content with tryptophan and indolelactate resulted in the production of IPA *in vitro* (Smith and Macfarlane 1997). *In vivo*, IPA was detected in the plasma and

cerebrospinal fluid (Young et al. 1980). Intriguingly, IPA was shown to completely protect primary neurons and neuroblastoma cells against oxidative damage and death caused by exposure to Alzheimer β amyloid protein, via inhibition of superoxide dismutase, or by treatment with hydrogen peroxide (Chyan et al. 1999). Collectively, the gut microbiota appears to sequester tryptophan from the diet and alter its metabolites in the host, resulting eventually in altered brain levels of neuropeptide that affect the brain function. In fact, the key microbial enzyme tryptophan decarboxylase, which converts dietary tryptophan to the neuropeptide tryptamine, has been recently identified (Williams et al. 2014). The enzyme was found to be present in several bacteria that colonize about 10 % of the human population. Altered levels of tryptamine in urine have been used in diagnosis, where low levels of tryptamine in urine were detected in patients with severe depression (Coppen et al. 1965). Tryptamine also stimulates the release of serotonin from enterochromaffin epithelial cells (Takaki et al. 1985) and is a key regulator of the gut motility and secretion (Turvill et al. 2000).

VITAMINS B12 and K are synthesized by the intestinal microbiota that are also capable of producing most of the water-soluble B vitamins, such as biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin and thiamine in humans (Hill 1997). Vitamin B12 (cobalamine), which is essential for the development of the nervous system (Dror and Allen 2008), is produced by *L. reuteri* (Santos et al. 2008). Vitamin K, which has a modulatory role in cognition (Ferland 2012), is produced by several bifidobacterial strains (Leblanc et al. 2013). Microbially produced vitamins are taken up in the colon unlike the dietary vitamins, which are adsorbed in the proximal tract of the small intestine (Said and Mohammed 2006).

SCFAs are produced by the fermentation of complex polysaccharides in the colon, which require the cooperative action of different microbial population groups, including various species of the anaerobic Fermicutes; Clostridial clusters IV and XIVa such as *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus* species (Flint et al. 2012; El Aidy et al. 2013c). Complex carbohydrates are broken down to mono- and oligomeric compounds that can be broken down further to the SCFA acetate, propionate, and butyrate as well as to carbon dioxide (CO_2,) and molecular hydrogen (H_2). Other important intermediates are lactic acid, ethanol, succinic acid, and formate that are also degraded to SCFA, CO_2, and H_2 (Blaut and Clavel 2007). SCFAs are the preferred source of energy for colonocytes and are also considered as a source of energy to the brain, where they can cross the blood-brain barrier to be taken up by the glial cells and, to lesser extent, the neurons (Karuri et al. 1993). Subsequently, continual secretion of SCFAs by the intestinal microbiota may result in long-lasting effects on gene expression patterns that are necessary for appropriate neuronal development and function even though the effects of SCFAs that cross the blood-brain barrier under physiological conditions may be marginal.

In adequate levels, propionate was shown to improve insulin sensitivity and lower serum cholesterol levels (Hosseini et al. 2011). Nonetheless, studies conducted in rodents suggested that excessive levels of propionate are detrimental to the host health and behaviour, indicating it is crucial to maintain a balanced microbial community. Indeed, higher levels of Bacteriodetes and Clostrial species were detected in patients with ASD (Hosseini et al. 2011). Intraventricular injection of propionate in rats was associated with impaired social behaviour and altered brain

phospholipid composition in a way similar to symptoms observed in ASD patients (Thomas et al. 2012a). The link of propionate with ASD is due to its ability to alter the levels of the neuropeptides; serotonin, glutamate and dopamine (El-Ansary et al. 2012). Butyrate has been the focus of many *in vitro* and *in vivo* studies, which unravelled its impact on the human physiology. Butyrate regulates energy homeostasis, stimulate leptin production in adipocytes (Musso et al. 2011). Moreover, levels of the neuropeptide glucagon-like peptide-1 (GLP-1) were induced in response to butyrate, resulting in modulation of insulin secretion, lipid and glucose metabolism, and food intake (Burcelin et al. 2007). Butyrate was also reported to have profound effects on mood and behaviour, where it elicited antidepressant effects in murine brain (Schroeder et al. 2007). Through their effect on gastric motility and intestinal transit stimulation, SCFAs resulted in an elevation in serotonin release as reported in an *in vitro* colonic mucosal system (Grider and Piland 2007). SCFAs have been reported to alter the expression of brain derived neurotrophic factor (BDNF), stimulate the sympathetic nervous system, and may influence social behaviour in rodents (Macfabe et al. 2011; Schroeder et al. 2007). Acetate, is a major substrate for acetyl CoA synthesis and, through the histone acetyltransferase (HAT) activity, is involved in the process of acetylation of histone tail lysine residues (Stilling and Fischer 2011). Thus, SCFAs enhance histone acetylation by inhibition of HDACs on the one hand and increased availability of HAT substrate on the other hand. In fact, several *in vitro* and *in vivo* models for learning and memory and neurodegenerative diseases illustrated that enhanced histone acetylation facilitated long-term memory consolidation and neuroprotection/-regeneration in a numerous *in vitro* studies and animal (Peleg et al. 2010; Govindarajan et al. 2011).

In summary, the microbial derived SCFAs are essential contributors to host metabolism through their action as an energy source or through balancing host gene expression throughout brain development and, more dynamically, in adulthood (Macfabe 2012; Gundersen and Blendy 2009; Thomas et al. 2012b; Selkrig et al. 2014).

In addition to SCFAs, the intestinal microbiota is capable of affecting the availability of dietary sources of methyl-group donors by modulation of one carbon metabolism and thereby potentially affecting host DNA and histone methylation (Cabreiro et al. 2013). Spermidine, a ubiquitous polyamine, has also been also shown to be produced by the intestinal microbiota (Noack et al. 2000). Spermidine has beneficial effects on ageing and age-associated memory impairment (Gupta et al. 2013), which may in part be mediated by an alteration in histone acetylation (Das and Kanungo 1979).

15.4.1.1 Neuroactive Chemicals

It is well recognized that a variety of the intestinal microbiome, in particular, lactic acid bacteria, has the capacity to produce several molecules with neuroactive function including gamma amino butyric acid (GABA), acetylcholine, catecholamines, and serotonin. For example, GABA is produced by species of Lactobacillus and Bifidobacterium, with *L. brevis* and *B. dentium* being the most effective producers (Barrett et al. 2012). GABA appears to protect its producing bacteria from the

gastric acidity (Higuchi et al. 1997). Intriguingly, *in vivo* experiments illustrated a modulatory effect of the microbiota-derived GABA on the host neural cells (Bravo et al. 2011). Ingestion of *L. rhamnosus* JB-1 resulted in differential expression of the GABA A and B receptor subunits, which are responsible for maintaining normal fear and mood responses.

Several species of *Escherichia*, *Bacillus*, *Lactococcus*, *Lactobacillus*, *and Streptococcus* are capable of producing the catecholamines; dopamine and norepinephrine (Shishov et al. 2009) in quantities that are thought to be higher than the catecholamines content in the human blood (Wall et al. 2014). The intestinal lumen of GF mice has lower levels of biologically inactive dopamine and norepinephrine than their conventionally raised counterparts. The substantial elevation of the luminal free catecholamines was associated with the colonization of gut microbiota, which has abundant beta-glucuronidase activity (Asano et al. 2012). Moreover, dopamine receptor expression is significantly altered in the gut and circulation but also in the brain during development (El Aidy et al. 2013b; Diaz Heijtz et al. 2011; Neufeld et al. 2011). Catecholamines represent the major class of neurotransmitters, which are involved in various neurological functions including emotion and endocrine regulation as well as cognition and memory processing (Kobayashi 2001). Subsequently, disturbance in catecholamines levels have been linked to several neurological disorders such as Parkinson disease (Calabresi et al. 2013), Alzheimer's disease (Robertson 2013) and major depressive disorders (Hamon and Blier 2013).

Acetylcholine is produced by different strains of Bacillus and Lactobacillus, in particular *L. plantarum* (Girvin and Stevenson 1954). Acetylcholine is crucial in maintaining cognitive function, memory and learning as well as the neuroinflammatory influx circuit (Olofsson et al. 2012).

Various Streptococcus, Escherichia, Enterococcus, Lactococcus, and Lactobacillus strains produce the neuroactive compound serotonin. Reports from animal experiments showed that ingestion of *B. infantis* for 2 weeks resulted in elevated plasma levels of the precursor of serotonin; tryptophan (Desbonnet et al. 2008). Moreover, the plasma levels of serotonin were three times higher in conventionally raised mice when compared to GF mice (Wikoff et al. 2009). In contrast, the levels of tryptophan were lower in conventionally raised mice (El Aidy et al. 2014a; Clarke et al. 2013). It remains to be unfolded how the intestinal microbiome impacts the tryptophan metabolism, presumably through the modulation of the expression level of the catalytic enzyme indoleamine 2,3 dioxygenase (Ido) in the Kynurenine arm, which occurs during immune activation (Moffett and Namboodiri 2003) and is observed in many disorders of both the brain and GI tract (Forsythe et al. 2010) but also transiently during primary gut colonization (El Aidy et al. 2012a). Recently it has been shown that gut microbiota, acting through SCFAs, is an important determinant of enteric serotonin production and homeostasis (Reigstad et al. 2014). The gut microbiota from ex-GF colonized with human gut microbiota and conventionally raised mice significantly increased the colonic rate limiting for mucosal serotonin synthesis; tryptophan hydroxylase (Tph) 1 mRNAs as well as the neuroendocrine secretion gene; chromogranin A, through the action of SCFAs.

Cyclic dipeptides (CDPs), a group of hormone-like compounds, represent another example of inter-kingdom signalling. Bacteria not only use CDPs in communicating with each other, but also in signalling to its host that uses the same molecules to regulate inflammation and induce protective effect in neuronal cells (Bellezza et al. 2014), suggesting that CPDs could have therapeutic value in a range of inflammatory and neuronal disorders.

It remains to be elucidated though why certain members of the intestinal microbiome are capable of producing neurochemicals. Bacteria use these molecules to communicate with each other in a process known as Quorum-sensing (Lyte 2011). It is thus possible that bacteria utilize these chemical signalling molecules in order to communicate with their hosts (Boontham et al. 2008). In fact, these intraluminal neuropeptides are thought to play a crucial role in modulating the ENS through their action on epithelial cells, to eventually influence the central nervous system (CNS), mood and behaviour (Lyte 2011; Dinan et al. 2013). It is thus tempting to view these bacteria as delivery vehicles for neuropeptides, which may be pivotal in the prevention and treatment of certain neurological and psychological disorders. This idea has recently been coined the term "psychobiotics" (Dinan et al. 2013).

15.4.2 Microbial Mimicry of the Host Epigenetic Machinery

Next to modulation of host epigenetics through metabolic activity, several pathogenic bacteria are able to secret effector proteins that mimic eukaryotic epigenetic enzymes and regulators to orchestrate infected cells in their own favour (Bierne and Cossart 2012). So far, these molecular tools have been discovered exclusively in intracellular parasites that interact with host signalling within the intracellular environment. It will be intriguing to search for such capabilities neuron-targeting pathogens as well as non-pathogenic microbes secreting epigenetic modulators that may be transported across epithelia and plasma membranes to affect host cells in a more paracrine manner. Taken together, the above-mentioned examples for inter-kingdom molecular manipulation shows the versatile ways open to microbes to interact with the host's transcriptional machinery.

15.5 The Gut-Brain Axis: The Pathways for Gut Microbiota to Modulate Brain Function

Although great progress has been made over the last decades in describing the bidirectional interactions between the GI tract, ENS and CNS, novel interest in this field of research has been stimulated by a growing body of intriguing preclinical studies signifying a prominent role of the gut microbiome in the gut-brain dialogue. The modulatory effect of the gut microbiota on the gut brain axis was demonstrated

in a number of studies using GF animal models, or modulation of microbiota with antibiotics, faecal microbial transplantation (Bercik et al. 2011a) or probiotics (Bravo et al. 2011). On the molecular level, GF mice show an altered expression of genes involved in neuropeptide production, *N*-methyl-D-aspartate (NMDA) receptor subunits (Neufeld et al. 2011) and genes involved in brain development and behaviour including altered expression of BDNF in the hippocampus (Diaz Heijtz et al. 2011; Gareau et al. 2011). Interestingly, some of the reported molecular changes in neuroreceptor expression have been associated with altered mood and behaviour. In the absence of a normal gut microbiome, significant changes in adult depression like behaviour (Schroeder et al. 2007), nociceptive responses (Amaral et al. 2008; Rousseaux et al. 2007), stress responsiveness (Gareau et al. 2007) and social development (Desbonnet et al. 2014) have been shown, and these alterations were partially reversed by colonization of the GI tract. The gut microbiota was associated with elevation in anxiety-like behaviour in conventional mice upon ingestion of probiotics such as *L. rhamnosus* (Bravo et al. 2011), *B. longum* (Bercik et al. 2011a) and *B. infantis* (Desbonnet et al. 2010). GF mice have recently been shown to have elevated repetitive behaviours and core social abnormalities in a similar way to that observed in ASD (Desbonnet et al. 2014). These findings are further supported by the correlative studies performed in humans, albeit in relatively small cohorts, which suggested that ASD may be associated with alterations in microbiota composition and metabolism (Critchfield et al. 2011; De Theije et al. 2011; Gondalia et al. 2012; Louis 2012; Macfabe 2012; Ming et al. 2012; Mulle et al. 2013; Douglas-Escobar et al. 2013). As such, the mechanisms underlying the development of ASD are still to be determined. While the cause of these conditions seems to be mostly genetic (including *de novo* mutations), it is unclear how the genetic information is translated to the behavioural and gastro-intestinal phenotypes associated with ASDs. Interestingly, both epigenetic mechanisms, including ncRNAs, (Grafodatskaya et al. 2010; Hall and Kelley 2014; Helsmoortel et al. 2014; Mellios and Sur 2012; Miyake et al. 2012; Schanen 2006; Wilkinson and Campbell 2013; van de Vondervoort et al. 2013; Ziats and Rennert 2013) and changes in the microbiota (Cao et al. 2013; Desbonnet et al. 2014; De Theije et al. 2014; Hsiao et al. 2013; Gorrindo et al. 2012; Kang et al. 2013; Peters et al. 2014) have been suggested to be involved in this process (Stilling et al. 2014a).

Additionally there is evidence for a contribution of genetic and environmental risk factors as well as a strong effect of microbial composition in visceral hypersensitivity associated with IBS (Ford et al. 2014; Fukuda and Ohno 2014; Shankar et al. 2013). Probiotic-based therapies have been used to decrease visceral hypersensitivity in preclinical models (McKernan et al. 2010) and in human trials (Clarke et al. 2012). Moreover, in line with the suggestion that epigenetic mechanisms are at the heart of the clinical manifestation of IBS (Dinan et al. 2010) Greenwood Van-Meerveld demonstrated amelioration of the stress-induced increase in visceral pain sensitivity by administering the epigenetic drug TSA (an HDAC inhibitor) directly to the brain (Tran et al. 2013). Future studies should be targeted at clarifying how microbes and their metabolites modify epigenetic pathways relevant to the origin and central processing of visceral pain.

Much of the attention directed towards the gut microbiota in regard to the gut-brain axis focuses on the ability of these commensals to potentially recruit the HPA axis to regulate the stress response and studies performed in GF mice illustrated an increase in the activity of HPA axis as measured by blood corticosterone or adrenocorticotrophic hormone levels (Neufeld et al. 2011; Clarke et al. 2013; Sudo et al. 2004). In contrast, gut neuropeptides have received considerably less attention in this regard although they can both be controlled by and influence the activity of the microbiome. Neuropeptides are produced in the gut in response to the microbial residents (Lyte and Cryan 2014), analogous to the neuroactive chemicals produced by the intestinal microbiota as discussed earlier. Specific members of gut neuropeptides can function at multiple levels of the brain-gut axis to guide not just local events in the GI tract but also distally at the level of the CNS to influence brain and behaviour. Bacteria can actively recognize the host neuroendocrine hormones and several studies showed the rapid growth and enhanced surface attachments (through biofilm formation) of these microorganisms when cultured in media containing small amounts of the catecholamines (Lyte et al. 2003). These findings were the groundwork of the theory of "microbial endocrinology". Intriguingly, when mice were administered a neurotoxic drug that caused the release of norepinephrine from the catecholaminergic neurons in the gut and other autonomic sites, the number of the gut populated gram negative bacteria are massively increased. These changes reversed as the catecholaminergic nerves regenerate within 2 weeks. The emerging evidence of the dramatic impact that the altered neuroendocrine environment can have on the gut microbes suggest that even minor changes in the levels of neuropeptides can lead to dysbiosis.

Bacterial components and by-products that come in contact with the gut epithelium stimulate a group of gut epithelial cells, enteroendocrine cells (EECs), to produce several neuropeptides such as peptide YY, neuropeptide Y, cholecystokinin, glucagon-like peptide-1,2, and substance P (Furness et al. 2013). Through receptors expressed on EECs, neurons of submucosal and myenteric ganglia as well as on enteric leukocytes, the gut senses the bacterial by-products (Samuel et al. 2008; Nohr et al. 2013). Upon their secretion by EECs, neuropeptides presumably diffuse throughout the lamina propria, which is occupied with a variety of immune cells, until they reach the blood stream or act on the vagal nerve or intrinsic sensory neurons (Cummings and Overduin 2007; Okano-Matsumoto et al. 2011) but the exact mechanisms and whether the neuropeptides have a direct contribution in the bidirectional communication between the microbiota and CNS are still obscure. Recently, Bohorquez et al. suggested an accurate temporal transfer of the sensory signals originating in the gut lumen with a real-time modulatory feedback onto the EECs. They demonstrated a direct communication between EECs and neurons innervating the small intestine and colon that alternates the paracrine transmission (Bohorquez et al. 2015).

The interplay between gut microbiota and the gut-brain axis can also occur through the autonomous (enteric, sympathetic and parasympathetic, which includes the vagus nerve) nervous system (ANS), sensory nerves, immune mediators and alterations in the gut functionality (motility, secretion, and permeability) (Mayer 2000; Holzer and Farzi 2014).

The ANS was shown to influence the size and quality of the mucus layer, and subsequently on the biofilm structures, where the majority of the colonic microbiota reside (Rhee et al. 2009). Throughout the GI tract, the community structure of commensals is likely to be dramatically altered in response to disturbed gut motility and increased intestinal permeability, which is also associated with small intestinal bacterial overgrowth (SIBO) (Van Felius et al. 2003). GI dysfunction, in particular constipation, often precedes the onset of motor symptoms by years in the aetiology of Parkinson's disease (PD), since both ENS and parasympathetic nerves are amongst the structures earliest and most frequently affected (Derkinderen et al. 2011). A recent study carried out in PD patients illustrated that the intestinal microbiome is altered in PD and is related to motor phenotype (Scheperjans et al. 2014). When the faecal microbiomes of 72 PD patients were compared to those of 72 control subjects, the abundance of Prevotellaceae was found to be dramatically reduced in faeces of PD patients. Moreover, the relative abundance of Enterobacteriaceae was positively associated with the severity of postural instability.

Several of the studies investigating the impact of the intestinal microbiota on behavioural and neurophysiological changes investigated the contribution of the vagus nerve. Indeed, vagotomy eliminated some of the effects found in studies on mice fed with probiotics or pathogens (Bravo et al. 2011; Bercik et al. 2011b). The modulated stimulation of the vagal pathways could occur as a result of altered gut motility or neurochemicals produced by the intestinal microbiota. Nonetheless, the exact modalities of how the vagus interacts with the microbiota to induce such effects are obscure. Other experiments suggested also that at least some of the observed effects of the gut microbiota on behavioural changes are functionally independent of the vagus or other autonomous pathways (Bercik et al. 2011a). Collectively, these findings indicate that the vagus nerve is an important, but not the only mediator in the microbiota-gut-brain axis.

15.6 The Microbiome as a Pivotal Component in the Psychoneuroimmunology Network

The intersection of neurology and immunity has its roots in modern science and the involvement of the nervous system in regulating the whole immune system and *vice versa* has led to the establishment of the field of psychoneuroimmunology (Ader and Kelley 2007). A growing body of evidence shows a significant contribution of immune signalling in normal brain function as well as during ageing and in the context of neurodegenerative diseases. This bidirectional cross talk is regulated by a network of signalling pathways, which involves (but not exclusively) the HPA axis and the ANS (Soliman et al. 2013; Lampron et al. 2013; Villeda et al. 2011; Collins et al. 2012). However, we are only beginning to fully appreciate the widespread interaction of the intestinal microbiota with the immune system and the neuroendocrine system, which strongly suggest the microbiota to be a decisive component in the psychoneuroimmunology network (El Aidy et al. 2014b). Intestinal microbiota may coordinate the neuroendocrine-immune dialogue via several mediators

including epithelial cells, (mucosal) immune cells as well as peripheral neurons (Forsythe and Kunze 2013; El Aidy et al. 2012b). For example, the ANS affects epithelial mechanisms involved in the intestinal immune activation through stimulation of the immune cells residing in the lamina propria to produce antimicrobial peptides against the enteric bacteria or via modulating the access of the microbiota to the immune cells (Alonso et al. 2008). The latter effect has been linked to changes in the gut permeability and increased translocation of commensals under stressful conditions (Keita et al. 2010). In fact, the context of the "leaky gut" hypothesis is another mechanism for inducing immunomodulatory effects in disorders of the brain-gut axis that involves the intestinal microbiota. Chronic stress, for example, has been shown to increase the gut permeability to bacterial peptides (Santos et al. 2001) and the adverse effects were reversed by probiotics (Ait-Belgnaoui et al. 2012; Zareie et al. 2006). These findings were supported by data from human studies, which indirectly suggest increased bacterial translocation in stress-related psychiatric disorders such as depression (Maes et al. 2012).

In healthy conditions, the majority of the gut microbiota is kept at bay, with no direct contact with the host cells, suggesting that the microbial products and neuroactive chemicals are most likely responsible for transferring the microbial signalling to the host. However, in states of disease and microbial dysbiosis, several pathobionts (potentially pathogenic bacteria, which are part of the normal microbiome community in a state of homeostasis) are capable of invading host tissues and can even live in intracellular vacuoles to manipulate host cells directly, thereby activating the mucosal immune cells and associated parts of the ENS (Lievin-Le Moal and Servin 2013). In fact, a similar mechanism could be orchestrating the early life modulation of the immune-neuroendocrine network following microbial colonization. Some animal studies illustrated that specific pathobionts activate mucosal immune process for bacterial sampling by transient breaching the epithelial barrier in order to minimize their exposure to the systemic immune system. This initial process of immune activation is associated with stimulation a variety of immune pro-inflammatory and regulatory immune components as well alter the expression of intestinal neuropeptides (El Aidy et al. 2013b, 2014a; Galindo-Villegas et al. 2012; Mazmanian et al. 2005). Elimination of the penetrant bacteria would require the engagement of immune and neuroendocrine components. Hence, this initial close but regulated contact could benefit the host by strengthening its gut barrier and conferring protection against true invading pathogens. Intriguingly, microbiota-immune-neurological communication is thought to be directed by neuropeptides, where many immune cells travel through the blood and when they come within scenting distance of a given neuropeptide they begin to chemotaxically orient toward it, and then communicate with other immune cells (Straub et al. 2006). Indeed, several neuropeptides direct the migration of immune cells, including immature DCs migration to lymph nodes at the start of a local immune response, as well as during the initial microbial colonization, via α1 adrenergic receptors, emphasizing the early involvement of the SNS (Maestroni 2000). The interaction between specific type of immune cells, ENS and microbiota was recently shown to

also regulate the GI motility (Muller et al. 2014). This regulation occurs when a subset of macrophages, named muscularis macrophages (MMs) that reside in close proximity to the myenteric plexus and intestinal cells of cajal (ICC) is stimulated by the intestinal microbiota to produce signalling molecules, which in turn stimulates the ENS (Kunze and Furness 1999; Muller et al. 2014). Whether the regulatory mechanisms employ signals provided by EECs remains to be elucidated.

Stimulation of the immune response by the gut microbiota induces the production of pro-inflammatory cytokines, which can inhibit the release of norepinephrine from noradrenoceptor axon terminals, via the induction of nitric oxide (Ruhl and Collins 1997). Elevated immune responses have also been linked with decreased activity of L-DOPA decarboxylase, the enzyme that converts L-DOPA to norepinephrine, and subsequently reduced levels of norepinephrine as observed in both inflamed and non-inflamed colonic mucosae of Crohn's patients (Magro et al. 2002). In fact, increased concentrations of inflammatory cytokines are known to circumvent the mechanisms of action of conventional antidepressants, suggesting that inhibition of the inflammatory cytokines would reduce depressive symptoms. This assumption was supported by the results from a placebo-controlled, randomized clinical trial showing that the TNF antagonist infliximab reduces depression symptoms in a subset of patients with high baseline inflammatory biomarkers (Raison et al. 2013).

Moreover, immune stimulation with non-pathogenic bacteria was shown to activate a functionally and anatomically distinct subset of serotonergic neurons, in the interfascicular part of the dorsal raphe nucleus of mice, which are different from the subset of serotonergic neurons activated by anxiety-inducing stimuli or uncontrollable stressors (Lowry et al. 2007). Activation of the peripheral immune system with antigens derived from the nonpathogenic bacterium *Mycobacterium vaccae* resulted in increased serotonin metabolism within the ventromedial prefrontal cortex, with temporal reductions in immobility in the forced swim test, indicative of alters stress-related emotional behaviour. Likewise, treatment with serotonergic antidepressant drugs prevents the onset of depressive symptoms in patients with irritable bowel syndrome (IBS), and in patients receiving treatment with interferon (Capuron and Miller 2004; Felger et al. 2013). Collectively, these findings suggest that serotonergic systems may be a plausible route by which the gut microbiota coordinates the immune-neuroendocrine communication. Whether the results to date reflect a causative or reactionary response is yet to be elucidated.

The principle vagal neurotransmitter, acetylcholine, is another important mediator in shuttling information between the microbiota and host nervous and immune systems, all of which can produce and respond to neuropeptides. Acetylcholine attenuates the release of a plethora of pro-inflammatory cytokines with no effect on the anti-inflammatory IL-10 (Borovikova et al. 2000), and is released from a subset of CD_4^+T cells that transfer the signal to other immune cells through the activation of α7 nicotinic acetylcholine receptors on macrophages (Wang et al. 2003). Acetylcholine is also produced by a specific type of T cells known as $ChAT^+$ T cells that are present in high abundance in the Peyer's Patches, supposedly to exhibit both

defensive and regulatory roles at the gut mucosal surface, where trillions of microbes reside (Rosas-Ballina et al. 2011). Microbial colonization was shown to be required for the expression of a subset of B cells that expresses acetylcholine receptor ($ChAT^+B$ cells), which reside only in mucosal-associated lymphoid tissues (Reardon et al. 2013). Expression of the acetylcholine receptor on $ChAT^+B$ cells begins at birth and involves MyD88 dependent toll like receptor signalling. The assumption was evident by the reduction in Ach receptor expression following antibiotic treatment. Taken together, this evolving data strongly supports a key role of the gut microbes in orchestrating psychoneuroimmunology functions.

15.7 From Animals to Humans: Translation of Laboratory Animal Research Evidence

Despite the extensive preclinical data supporting the impact of the gut microbiota on the gut-brain dialogue, limited information is available of how these findings may translate to human in health and disease. A recent study in a healthy cohort of 56 vaginally born Dutch infants was performed to investigate the development of the gut microbiota as a potential pathway linking maternal prenatal stress and infant health (Zijlmans et al. 2015). The findings showed clear links between maternal prenatal stress and the infant gut microbiota. Infants of mothers with high cumulative stress during pregnancy had significantly higher relative abundances of the pathogenic Escherichia, Serratia, and Enterobacter, and lower relative abundances Lactobacillus, Lactoccus, Aerococcus and Bifidobacteria. The disturbed colonization pattern illustrated in this study was related to infant gastrointestinal symptoms and allergic reactions suggesting a role of elevated level of inflammation. Another study conducted in infants with colic demonstrated an overall reduced diversity of the gut microbiota with an increase in Proteobacteria and decrease in Bacteroides in comparison to healthy infants (De Weerth et al. 2013). Although the causal role of microbial dysbiosis in these studies remains elusive, the association between disturbance in microbial composition and clinical phenotypes suggest a potential effect on bacterial interventions on behaviour and health. Indeed, in a placebo-controlled study of healthy women, brain changes in response to a fermented milk product containing four different probiotic interventions were assessed by functional magnetic resonance imaging. This study suggested a basic change in responsiveness to negative emotional stimuli (Tillisch et al. 2013). Brain imaging was also applied in another study conducted in patients with hepatic encephalopathy, mild cognitive disorders, and antibiotic treatment to alter the microbiota composition (Bajaj et al. 2013). Antibiotic treatment resulted in improved cognitive functions and changes in blood fatty acids metabolites that were suggested to be of microbial origin. Even though no changes in the microbial abundance were reported in the study, the results suggested that the improved cognition could be attributed to a shift from pathogenic to beneficial metabolites.

15.8 Conclusions

It is now well established that "healthy" mammalian structure and function is significantly dependent on its residing microbes. Data from animal studies clearly illustrated that unfavourable alterations in the body's organ systems are linked with microbiota dysbiosis. However, we need a better understanding of the causative mechanisms whereby these interactions occur in order to provide novel avenues to rationally intervene in disease situations with either microbial or dietary interventions that aim to correct imbalance situations and thereby restore homeostasis. To date, limited information is available of how the findings from animal experiments may translate to human in health and disease. The enormous amount of inter-individual variation observed in the microbial composition and its genome require the application of very large studies to distinguish disease-associated changes, which renders the broad application of these trials very complicated within the human population. Moreover, the wide array of variation among individuals in terms of diet, genetics, environmental factors and sex-related differences, adds to the complexity of the human microbiome analysis. Practical and ethical concerns associated with the use of human volunteers represent another challenge that limits clinical intervention studies even with probiotics and if allowed, such studies are generally conducted only in primarily healthy individuals and not the human population that is most at risk.

The expanding repertoire of diseases associated with microbial dysbiosis urges the need to explore what our microbes do and not remain at a stage of describing who they are. Undoubtedly, the major advances in metagenomic and metabolomics technologies are continuing to help the reconstruction of metabolic pathways, which revealed that the microbiome functional diversity is by far less than its genetic diversity, with respect to the inter-individual variation (Human Microbiome Project Consortium 2012). A better understanding of the functionality of the intestinal microbiota will not only help to design future probiotics but also to genetically engineer the microbiome in a way that produces microbial metabolites, which are beneficial to the host or could be applied as nutraceuticals. Moreover, deciphering the dialogue among the microbial community would allow manipulating the community, particularly in susceptible individuals, in a way to remove those bacterial species representing a threat to the balanced microbial consortia, without necessarily being a pathogen.

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Chapter 16
Mycologic Endocrinology

Karl V. Clemons, Jata Shankar, and David A. Stevens

Abstract The interactions of fungi and chemical messenger molecules, hormones or pheromones, are addressed in this chapter. These interactions include mammalian fungal pathogens, also plant pathogens, or non-pathogenic fungi, which can result in functional responses in receptor- or non-receptor-mediated fashions. Endogenous ligands in the fungi have been demonstrated to be important for mating in a number of systems. Mammalian hormones have been demonstrated to have stimulatory or inhibitory effects on growth for organisms such as *Candida albicans*, *Paracoccidioides brasiliensis*, *Saccharomyces cerevisiae*, *Rhizopus nigricans*, *Aspergillus fumigatus*, *Coccidioides*, and dermatophytic fungi. A number of fungi have been shown to have specific binding proteins for corticosteroid, estrogen and progesterone that are stereo-specific and high affinity. In some instances, the interactions of a mammalian hormone with the organism, *in vivo,* affects pathogenesis. Genome expression profiles of *C. albicans* in the presence of estradiol or progesterone, and *S. cerevisiae* with progesterone, indicate major up-regulation of various drug resistance pumps, like *CDR1,* and *CDR2,* can affect antifungal susceptibility. Azole antifungal interactions occur with fungal hormone binding proteins. Azoles also can block mammalian steroidogenesis. The finding of interactions of mammalian hormones with fungi and subsequent functional responses by the fungi, suggest that hormonal interactions with fungal systems has been conserved throughout evolution and have an important role in fungal pathogenesis, as well as in the overall biology of the organisms.

Keywords Fungi • Drug effects • Hormones • Glucocorticoids • Growth and development • Metabolism

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16.1 Introduction

Hormones serve as regulatory messenger molecules, inducing regulation of gene-expression through receptor-mediated interactions, and the subsequent functional response to the presence of the hormone. Molecules considered to be hormones include steroids, such as 17β-estradiol (E_2), progesterone, and testosterone, and protein hormones such as insulin, human growth hormone and luteinizing hormone (LH).

The present understanding of the action of hormones is derived from mammalian systems, where hormones regulate a variety of functional processes in a broad range of tissues. Target cells are affected in a tissue specific manner after binding of the hormone by a specific receptor; the extent of the specific response is determined by the proteins, pathways, and processes with which the receptors interact, as well as the concentration of hormone present. Classically, hormone receptors (e.g., steroid receptors) act as transcriptional regulators in the nucleus, after the hormone-receptor complex moves across the nuclear membrane, where it binds to specific response elements in the DNA (Beato et al. 1996; Funder 1997; Whitfield et al. 1999; Walters and Nemere 2004). However, not all steroid receptors are located intracellularly and some are plasma membrane associated (Hammes 2003; Walters and Nemere 2004). In addition, steroids, and other hormones, can have nongenomic actions, such as modulation of MAP kinases or tyrosine kinases and regulation of membrane ion channels and G-proteins (Losel and Wehling 2003; Simoncini and Genazzani 2003).

Fungi also utilize hormones (sometimes called pheromones) as messenger molecules that regulate various activities of the organism. Primarily, these molecules are related to control of sexual reproduction in various fungi, and take the form of novel steroids, peptides, and acid derivatives (Gooday 1974; Gooday and Adams 1993). In addition, fungi produce secondary metabolites, such as zearalenone, which has high estrogenic activity in mammalian cells. Zearalenone, and its metabolites, α-zearalanol, β-zearalanol, α-zearalenol, and β-zearalenol, act as a trigger factor for the development of central precocious puberty and high growth rate of girls exposed to environmental sources of these mycotoxins (Massart et al. 2008; Massart and Saggese 2010). It has long been known that fungi can metabolize, transform and convert mammalian steroids (including the production of steroid molecules that mammalian cells cannot produce), which has proven useful in the biotechnology production field (Mahato and Garai 1997; Fernades et al. 2003; Kristan and Rizner 2012).

The present chapter will address different aspects of fungal endocrinology, particularly interactions of mammalian hormones with fungi. Two reviews have been written previously addressing in detail the work done in our laboratory and that of our collaborators (Feldman 1988; Stevens 1989). We will present an overview of this work, which focused on characterization of hormone receptors in fungi, as well as any physiological effect or functional responses induced by the presence of the hormone in the systems we studied. In addition, we will include more recent studies from other investigators addressing functional responses of fungi to mammalian hormones.

16.2 Hormones and Mating in Fungi

Sexual reproduction of several fungi is known to be under the control of hormones, which act as chemoattractants and also stimulate a functional response via a specific receptor for the target fungal mating type to become capable of mating with the opposite mating type (e.g., male and female, or + and − strains, or a and α strains) (Gooday 1974; Gooday and Adams 1993; Bardwell 2005; Jones and Bennett 2011). For instance, in the water mould *Allomyces,* swimming female gametes produce sirenin, which is a sesquiterpine that acts as a chemoattractant for the male gametes, causing them to swim toward the female, where eventual contact between the two initiates plasmogamy.

An extensively studied fungal hormone system is that of the oomycete water mould, *Achlya* (Gooday 1974; Riehl and Toft 1984, 1985; Riehl et al. 1984; Brunt and Silver 1986a, b; Brunt et al. 1990, 1998; Gooday and Adams 1993; Silver et al. 1993). This organism produces two steroidal hormones, antheridiol by the female and oogonial by the male that cause the switch from vegetative growth to the formation of mating specific structures. Antheridiol induces the formation of antheridia by the male, which grow toward the female cells and similarly, oogonial induces the formation of oogonial initials by the female cells. The organism has been demonstrated to have a high-affinity binding protein for antheridiol, with a K_d of 7×10^{-10} M and capacity of up to 2000 fmol/mg of protein (Riehl and Toft 1984; Riehl et al. 1984). Furthermore, this specific binding protein has been demonstrated to be a true receptor, modulating gene expression in the fungus and in particular hsp70 and hsp90 (Brunt and Silver 1986a, b; Brunt et al. 1990, 1998; Silver et al. 1993). Thus, in this system, specific binding of the hormone and subsequent receptor-mediated regulation of gene-expression is similar to the regulatory mechanisms of hormonal interaction in mammalian systems.

Saccharomyces cerevisiae produces two peptide hormones, a- and α-factor, which act on the reciprocal mating type to arrest vegetative growth and enable mating. Interestingly, the α-factor peptide has sequence similar to mammalian gonadotropin-releasing hormone and can also bind to the mammalian receptor, inducing pituitary cells to release LH (Loumaye et al. 1982). Another yeast producing mating type specific peptide hormone is *Rhodosporidium* (Miyakawa et al. 1985). *Tremella* (a basidiomycete) utilizes two pheromones, A-10 and α-13 (isoprenyl peptides), to induce mating of haploid cells (Gooday 1974; Miyakawa et al. 1984; Gooday and Adams 1993). In the zygomycetes, trisporic acids act on both + and − mating types to induce gametangial tropism and subsequent mating, but appear to not be species specific (Gooday and Adams 1993; Schimek and Wostemeyer 2009). Overall, it is likely that sexual reproduction of most fungi is regulated by the production of mating type hormones.

A reciprocal mating pheromone system has been described in the human pathogen *Candida albicans*. Naturally occurring a and α clinical isolates have been identified and can undergo mating. For these cells to become mating competent, they must first undergo transition from the white phase to the opaque phase.

This well-described phenotypic switching system involves changes in the expression of many genes, including many involved in mating (Bennett et al. 2003; Lin et al. 2011). Cells of the a mating type respond to the α pheromone by producing long polarized projections that can result in the mating process. During this, transcription of many genes is induced. Of note, several of the genes encode surface and secreted proteins implicated in *C. albicans* virulence.

16.3 Interaction of Fungal Hormones with Plants, and Plant Hormones with Fungi

Host-microbial interactions are also important in the plant kingdom, and these interactions affecting fungal infection may be mediated by hormones (Gogala 1991; Prusty et al. 2004). However, for the remainder of the chapter we will focus on mammalian-fungal hormonal interactions.

16.4 Interaction of Mammalian Hormones with Fungi

In addition to the action of fungal-produced hormones, several pathogenic fungi are known to interact and respond to various mammalian hormones. These interactions can influence the growth, and even the pathogenesis of the organism. In the following sections we will examine some of these interactions and effects on specific organisms, as well as potential implications for pathogenesis.

16.4.1 Hormone Influence on Growth of Fungi

Early studies with the interactions of fungi and hormones focused on the growth inhibitory effects of high concentrations (e.g., $>10^{-3}$ M) of the hormones. However, in some instances mammalian hormones are stimulatory to the growth of the organisms (Stevens 1989). Dermatophytic fungi (i.e., *Trichophyton, Microsporum,* and *Epidermophyton*) have been demonstrated to be inhibited by the presence of various steroids including androgens and progesterone (Capek and Simek 1971; Stevens 1989; Brasch and Flader 1996). Furthermore, hydroxylation of progesterone reduces the inhibitory activity (Capek and Simek 1971). The inhibition of *T. rubrum* and *E. floccosum* by androgens has been suggested as a reason that these organisms do not cause tinea capitis (Brasch and Gottkehaskamp 1992; Brasch and Flader 1996). In addition, a dematiaceous fungus is inhibited by the mammalian hormones progesterone and testosterone (Hernandez-Hernandez et al. 1995).

Interestingly, some fungi have been shown to have stimulated growth in the presence of mammalian hormones. For instance, two studies have reported the stimulation of the growth of *C. albicans* by dexamethasone (Khosla et al. 1978; Gupta et al. 1982)

and another reported the stimulation of adherence to buccal cells by dexamethasone, but inhibition by corticosterone (Ghannoum and Elteen 1987). Estrogens have also been reported to stimulate the growth of *C. albicans* (Gujjar et al. 1997; Zhang et al. 2000) and promote spherule growth and endospores release in *Coccidioides* (Powell et al. 1983). Furthermore, hydrocortisone has been shown to stimulate the growth of *Aspergillus fumigatus* and *A. flavus* (Ng et al. 1994). Overall, results showing growth stimulation by mammalian hormones in these studies have implications for enhancing or promoting the pathogenesis of these organisms.

16.5 Specific Steroid Binding Proteins in Fungi

As can be noted from the investigations noted above, in some organisms specific receptors for endogenous hormones have been demonstrated and various fungi interact with mammalian hormones. This raises the question of whether the fungi respond to the non-endogenous mammalian hormones via the binding of the hormone to a fungal protein, which acts as a receptor, or do fungi have specific receptors for mammalian hormones? These questions have been addressed for a number of fungal pathogens and are presented in the following sections.

16.5.1 Paracoccidioides brasiliensis

Paracoccidioides brasiliensis is a thermally dimorphic fungus, which causes the most prevalent systemic mycosis (paracoccidioidomycosis) in Latin America. The fungus exists in soil as a filamentous form, which transforms into a yeast form when it invades mammalian hosts or by a temperature switch from room temperature to 37 °C, in vitro. Infection is initiated after inhalation of conidia or mycelial fragments by the mammalian host. These mycelial propagules further differentiate into the yeast form of the organism, which is found in the tissues. The transition of mycelial to yeast form is crucial for the establishment of disease. Epidemiologically, clinical disease is more common in adult men than women despite equal frequencies of exposure to this fungus, and it has been estimated that females are about 13–70 times less likely as males to develop clinical disease (Brummer et al. 1993; Shankar et al. 2011a) Furthermore, the development of clinical disease is equal in males and prepubertal or post-menopausal females. Thus, this led to the speculation that human sex hormones might have an effect on progression of this disease.

Using a microculture system, morphological transformation of mycelia to yeast form of clinical isolates of *P. brasiliensis* was tested in the presence of various steroids, including 17β-estradiol (E_2), testosterone, tamoxifen and 17α-estradiol, and the non-steroidal estrogen, diethylstilbesterol (DES), at concentrations ranging from 2×10^{-10} to 2×10^{-6} M to span relevant physiological and pharmacological concentrations (Restrepo et al. 1984). Mycelial to yeast transformation was dose-dependently

inhibited by E_2 to 71 % (2×10^{-10}), 33 % (2×10^{-8}) and 19 % (2×10^{-6} M) of the transformation of control cultures. In addition, DES at these concentrations resulted in 85 % (2×10^{-10}), 54 % (2×10^{-8}) and 37 % (2×10^{-6} M) inhibition compared to control. Testosterone, tamoxifen and 17α-estradiol were inactive. Inhibition of the morphological transformation occurs only in one direction and none of the tested compounds blocked the transformation of yeast to mycelia nor did they affect yeast growth or budding. Similarly, the transformation of conidia to yeast was shown to be blocked by E_2 (Salazar et al. 1988). Thus, inhibition of transformation of conidia or mycelia fragments to yeast form is a biologically specific phenomenon with E_2 effective and its stereoisomer 17α-estradiol ineffective.

To identify a possible steroid hormone receptor in the cytosol of *P. brasiliensis,* yeast cells were grown and disrupted with glass beads to produce a cytosolic extract. Specific binding activity was examined by addition of [^{3}H]-E_2 and 500-fold molar excess of unlabeled E_2 to the cytosol to perform conventional steroid binding assays (Loose et al. 1983b). Bound hormone was separated from free hormone using centrifuged Sephadex microcolumns. In a single point study using 130 nM [^{3}H]-E_2, specific E_2 binding was demonstrated in the range of 200 fmol/mg of cytosol protein. Some specific binding was detected with [^{3}H]-progesterone (60 fmol/mg protein) and DES (42 fmol/mg protein); only trace binding was detected with dihydrotestosterone and testosterone, and no binding was seen with corticosterone. Progesterone binding was completely blocked by E_2, suggesting the presence of a single estrogen binding site with some cross reactivity for other steroids. However, the presence of a separate progesterone or DES binding site has not been completely excluded.

More extensive binding studies with cytosol and [^{3}H]-E_2 done at 0 °C indicated that binding was maximal after about 90 min of incubation (Loose et al. 1983b). The dissociation rate was determined and the off-rate time for half of the bound hormone to be released was 29 min. Binding characteristics at equilibrium showed binding to be saturable with low non-specific binding. Scatchard plots suggested a single class of non-interacting binding sites, with a dissociation constant (K_d) of 8.5 nM and a binding capacity (N_{max}) of 210 fmol/mg protein, indicative of high-affinity low capacity binding. The specificity of the binding was assessed in competition assays, where unlabeled hormones compete for specific binding with [^{3}H]-E_2. Competition for bound E_2 was maximal with E_2, whereas the related estrogens, estrone and estriol, had only about 25 % of the affinity of E_2. DES was a weak competitor, in contrast to its affinity for the mammalian E_2 receptor. Thermal stability studies at 0, 37, and 56 °C for over 30 min showed a second binding protein, with lower affinity at 37 °C. However, at 56 °C only the high capacity (N_{max} 1700–2600 fmol/mg protein) binding protein was detected. Treatments with DNase, RNase, and phospholipase A2 had little effect on the [^{3}H]-E_2 binding, whereas binding activity was inhibited by trypsin and reduced by *N*-ethylmaleimide. These results together suggested specific binding was due to a protein containing sulfhydryl groups. Liquid chromatographic (HPLC) studies indicated that the binding protein has a relative molecular mass of 60,000 Da and sucrose gradient centrifugation indicated a sedimentation coefficient of 4.4S.

Because the functional response of inhibition of form transformation was that of inhibiting the mycelial form, it was important to determine whether the mycelial form also contained a specific binding protein for E_2, and we were able to devise ways to work with the mycelial form of *P. brasiliensis* for binding assays (Stover et al. 1986). A binding protein was discovered in this form, in many isolates of the fungus, as had been shown for the yeast form. The K_d and N_{max} were 13 nM and 78 fmol/mg protein, respectively. In addition, a second low-affinity (K_d 150 nM) high-capacity (N_{max} 3000–4500 fmol/mg protein) binding protein was demonstrated in the yeast form. In competition studies, DES was a potent competitor for E_2 with the mycelial binder, correlating better with its functional effect of inhibiting the mycelium-to-yeast transformation described above.

The elucidation of a second mammalian estrogen receptor (Gustafsson 1999), with quite different ligand-binding domains, may necessitate re-examination of the putative estrogen receptor in fungi, particularly with receptor-specific ligands. We are in the initial stages of trying to determine the nature of the estrogen receptor in *P. brasiliensis* (Chen et al. 2013).

The mammalian estrogen system is complex (Katzenellenbogen and Katzenellenbogen 2000). The principal estrogen is E_2. In addition, the closely related molecules estrone (principally postmenopausal) and estriol (principally in pregnancy) are recognized; both are particularly potent against the nongenomic receptors. Finally, there are other metabolites or physiologic estrogens, which are weakly active against the genomic receptors but can be potently active with nongenomic receptors.

Several estrogen receptors are recognized. The nuclear, genomic estrogen receptors α and β have extensive homologies. Estrogen receptor (Er)α, the first described, tends to be the main driver of estrogen responses. Erβ can directly modulate Erα function. Erα and Erβ can form heterodimers, and splice variants of Erα and Erβ are recognized; these may differ in bioactivity from the wild type. In addition, there is a nongenomic cell membrane receptor, the G protein coupled estrogen receptor (GPER; formerly GPR30) (Soltysik and Czekaj 2013). Actions at Erβ and GPER tend to antagonize estrogen activities at Erα. Finally, there are orphan molecules termed "estrogen receptor-related receptors" whose actions are unknown, and some evidence there may be Erα and Erβ types in the membrane. Changes in signaling pathways can impact receptor function, as can growth factors.

In the search for estrogen blockers, driven largely by the problems of hormone responsiveness in breast and gynecologic cancers, compounds termed "selective estrogen receptor modulators" (Kieser et al. 2010) have been discovered that can act as agonists or antagonists of estrogens depending on the host tissue involved (e.g., tamoxifen). Compounds can act as agonists on Erα and antagonists on Erβ (e.g., hydroxytamoxifen). Endogenous co-regulator proteins can affect anti-estrogen (or hormone) expression, and thus activate or repress estrogenic activity, and Erα and Erβ can be differentially recruited. Drugs can change the phosphorylation state of receptors and thus modulate their function.

We seeded liquid cultures with *P. brasiliensis* mycelia, and added agents. We studied E_2; fulvestrant (Mehta et al. 2012), which binds mammalian Eαr and Eβr, antagonist to estrogen and down-regulator of the receptor, and mammalian

membrane receptor (GPER) agonist; and G1 (EMD Chemicals, Gibbstown, NJ), a mammalian estrogen agonist that competes with it at GPER, not binding to Eαr or Eβr. Concentrations of 10^{-6} to 10^{-8} M alone were studied. The test agents were studied individually and in combinations with E_2. In combinations E_2 was held constant at 10^{-7} M and the test agent added at the same molar concentration or tenfold less or tenfold greater. In some experiments the test agent was held constant at 10^{-7} M and E_2 added at the same molar concentration, tenfold less or tenfold greater. The culture temperature was switched to 37 °C to allow M to Y transition over 12 days. By day 12, cultures in all experimental groups moved in the direction of transformation to yeast, thus the "block" by estrogen or other agents described is really a retardation of M to Y shift. This could be related to the fungus biologically overcoming hormone or drug action, or hormone or drug metabolism, thermal inactivation, or absorption to the vessel wall.

In nine experiments, E_2 blocked M to Y transition as expected. Alone, G1 resembled a fungal estrogen agonist at high concentrations; fulvestrant had a small estrogen-like effect and was not dose responsive. G1 had a small antagonistic effect on estrogen at low (1:10) ratio, fulvestrant a small antagonistic effect at the lowest and highest ratios. In two experiments, diarylproprionitrile (Catley et al. 2008), a selective mammalian Eβr agonist had no effect on its own or on E_2's effect.

The prominent effect seen was G-1's estrogenic effect, alone, only at the highest concentrations. We therefore hypothesize that, functionally, the *P. brasiliensis* binding protein most resembles mammalian GPER in its activity, i.e., G-1 is an agonist in both mammalian and fungal cells.

The lack of dose-responsiveness in the mixing experiments make conclusions about G-1 or fulvestrant effects difficult. Conjectures about these interactions are confounded by the possible presence in the cultures of an endogenous *P. brasiliensis* molecule, which would be the true ligand for the identified *P. brasiliensis* binding protein, and could also compete with the added hormones or agents. We presume the *P. brasiliensis* binding protein has a role in the *P. brasiliensis* economy, and that its binding to hormones from mammalian creatures developed many layers of evolution later is a structural accident. What that role of *P. brasiliensis* binding protein-putative *P. brasiliensis* ligand interaction might be is unknown. We have attempted to define a molecule in *P. brasiliensis* culture filtrate, or even cytosol, with either molecular similarity to mammalian estrogen or with estrogenic activity in mammalian systems, without success to date (unpublished results), and it is possible that ligand has little to no resemblance to estrogen structurally (except perhaps at the binding site) or functionally.

Lack of dose-responsiveness is well-recognized in mammalian hormonal systems (Watson et al. 2011). So-called nonmonotonic and oscillating responses can be caused by parallel signaling through different receptors, with varying contributions from multiple pathways, resulting in inhibition or enhancement over a dose-response range. Hormesis is a term applied to one type of response curve, where inhibitory responses are triggered at hormonal concentrations above those which produce a maximal stimulatory response, and is thought to represent a safety mechanism to prevent overstimulation. Hormesis can occur as a result of effects triggered through different receptors opposing each other. In the mammalian estrogen system, another

contributor to the phenomenon of lack of dose-responsiveness is nongenomic estrogen regulation of enzymes, including the enzymes that produce estrogens or their metabolites. Finally, intracellular signaling is integrated at kinase nodes (e.g., MAP kinases), and the resultant process can have effects on downstream transcription factors, thus even possibly transforming nongenomic effects into genomic effects. Our studies of gene responses during E_2 block of M-Y suggested involvement of processes, such as stress responses, known to be affected by signaling pathways through kinase nodes (Shankar et al. 2011b).

16.5.2 Candida albicans

C. albicans is an opportunistic dimorphic fungal pathogen of medical importance. Studies by Loose et al. (Loose et al. 1981, 1983a; Loose and Feldman 1982), into the evolution of hormone-receptors, represents a classical example of mammalian steroid hormone interaction with the fungus. In those studies *C. albicans* was shown to have a protein capable of specific corticosteroid-binding (CBP) that exhibits high affinity for corticosterone and progesterone. Specific corticosterone binding was found in the cytosol of *C. albicans* with a K_d 6.3 nM and binding capacity N_{max} 650 fmol/mg of protein. Specific binding was demonstrated to be due to a protein with apparent molecular mass of 43 kDa (Loose et al. 1981; Loose and Feldman 1982). Interestingly, lipid extracts of the cell pellet or of culture filtrate displaced specific [^{3}H]-corticosterone from CBP, and are presumed to contain an endogenous ligand (Loose et al. 1981). Thus, CBP exhibits properties consistent with a receptor molecule. It is stereo-specific, extremely selective with affinity for corticosterone (~7 nM) that is equivalent to that of mammalian glucocorticoid receptors (Loose et al. 1981).

CBP has been found in both serotypes A and B of *C. albicans* and a survey of various species of *Candida* indicated that the binding protein appears ubiquitous within the genus (Loose et al. 1983a) and was confirmed in other studies (Powell and Drutz 1983). Although the binding parameters differed between species, Scatchard plots were linear, indicating a single class of binding sites in each. It was shown that corticosterone can enter an intact yeast cell and bind to the protein. *Candida* growth, yeast to mycelia conversion of *Candida* or glucose oxidation was not affected by the addition of different steroids (corticosterone, progesterone and dexamethasone) over a range of 10^{-6} to 2×10^{-10} M (Loose et al. 1983a).

To determine the relationship of CBP from *C. albicans* to the mammalian hormone receptors, the CBP gene has been cloned and expressed. It revealed an open reading frame of 1467 bp which encodes a protein with a molecular weight of 44,545 Da; the expressed protein has the properties of the native CBP. Sequence comparison of CBP gene to members of mammalian steroid-thyroid-retinoic acid receptor gene superfamily showed that CBP is unrelated to these hormone receptors (Malloy et al. 1993). Interestingly, in *S. cerevisiae*, the *FMS1* (fenpropimorph multicopy suppressor gene 1) yeast gene shows a protein identity of 35 % with *C. albicans* CBP (Joets et al. 1996).

C. albicans has also been shown to have an estrogen-binding protein (EBP) that displays high affinity for estradiol and estrone (Powell et al. 1984; Skowronski and Feldman 1989). Specific binding was found for E_2 with a K_d of about 6×10^{-8} M and N_{max} of 400–13,000 fmol/mg of protein depending on the study and the strain of *C. albicans* tested (Powell et al. 1984; Skowronski and Feldman 1989). Furthermore, the abundance of EBP was found to be significantly higher in early log phase growth (Skowronski and Feldman 1989). Binding was saturable and other estrogens, estrone and estriol, were the best competitors and unlike the situation of the human estrogen receptor, tamoxifen does not bind to EBP in *C. albicans* (Powell et al. 1984; Skowronski and Feldman 1989).

Cloning of the EBP gene revealed an open reading frame of 1221 bp that encodes a protein with 407 amino acids and having a molecular mass of 46,073 Da, the estimated size of EBP (Madani et al. 1994). The expressed gene showed high affinity with binding for estradiol and a competitive profile comparable to wild-type *C. albicans* EBP. Sequence comparison showed that EBP shares a 46 % amino acid identity with the old yellow enzyme, an oxidoreductase from *S. cerevisiae*, but, as anticipated, was unrelated to the human estrogen receptor. Expressed protein exhibited oxidoreductase activity and showed inhibition by the treatment of E_2 in vitro (Madani et al. 1994).

C. albicans has also been shown to interact with the human peptide hormones LH and human chorionic gonadotropin (Bramley et al. 1990, 1991). Both low and high affinity binding sites have been found for these peptides and the binding activity has been demonstrated in microsomes and cytosol preparations. Furthermore, LH was found to stimulate germ tube formation (Kinsman et al. 1988), and adenylate cyclase activity (Williams et al. 1990). In addition, other investigators have reported a human chorionic gonadotropin-like protein in extracts of *C. albicans* that was a potent stimulator of germ tube formation in the presence of serum (Caticha et al. 1993), which is suggestive that this protein is an endogenous ligand and regulator of germ tube formation.

16.5.3 Saccharomyces cerevisiae

Similar to *C. albicans*, *S. cerevisiae* has been demonstrated to have an EBP (Feldman et al. 1982; Burshell et al. 1984), as does *P. brasiliensis* as discussed earlier. The studies in *S. cerevisiae* showed it to have a 60–70 kDa protein that specifically bound E_2 with a K_d 1 nM with a binding capacity of 2000–4000 fmol/mg of protein. Interestingly, *S. cerevisiae* was thought to possess an endogenous estrogen ligand, but studies showed that the presence of estrone in the components of culture media (e.g., molasses and Bacto-Peptone) could be enzymatically converted to E_2 by the organism (Feldman 1988). The question of whether the EBP in *S. cerevisiae, Candida,* and *P. brasiliensis* are similar in sequence or activity is unknown.

16.5.4 Trichophyton *and* Microsporum

As noted earlier, the growth of dermatophytic fungi is inhibited by various steroid hormones. In addition, epidemiologic data are suggestive that men exhibit dermatophytic infections more frequently than women. Thus, we undertook a series of studies to investigate possible interactions with mammalian steroids.

Initial steroid binding studies were done with *T. mentagrophytes.* A specific binding of progesterone was demonstrated in the cytosol of the organism with a K_d 95 nM and binding capacity of close to 5000 fmol/mg of protein. Both deoxycorticosterone and dihydrotestosterone were strong competitors for progesterone binding, whereas other steroids had minimal binding activity (Schar et al. 1986). Furthermore, progesterone inhibited the growth of the organism, as did deoxycorticosterone and dihydrotestosterone, all in the same rank-order as for binding (Schar et al. 1986). Further studies showed the binding was due to a protein and that similar to *T. mentagrophytes*, *Microsporum canis* had a progesterone binding protein similar in affinity and high capacity, whereas the progesterone binding protein in *T. rubrum* was of higher affinity (i.e., K_d 16 nM) and lower capacity (i.e., 196 fmol/mg protein) (Clemons et al. 1988). Growth inhibition was demonstrated similar to the initial study, but this inhibition appeared to be a delay in growth from which the organisms escaped. Because *Trichophyton* had been reported previously to be able to metabolize progesterone (Capek and Simek 1971; Stevens 1989), we examined whether this might be an explanation for the escape from growth inhibition (Clemons et al. 1989b). Those results indicated that progesterone was metabolized to more polar and less growth inhibitory compounds including 15α-hydroxyprogesterone, 1-dehydroprogesterone, 11α-hydroxyprogesterone and 1-dehydro-hydroxyprogesterone (Clemons et al. 1989b). Thus, metabolism of progesterone is the likely mechanism for escape from the growth inhibition.

16.5.5 Coccidioides

The frequency of coccidioidal disease has been linked to gender, with males exhibiting clinical disease more frequently (Drutz and Huppert 1983). However, pregnancy, especially in the third trimester has been considered a risk-factor for the development of serious life-threatening disease. Studies by Powell et al. (Powell and Drutz 1984) showed that *Coccidioides* had a high affinity receptor for E_2 (i.e. K_d 21 nM, N_{max} 1500 fmol/mg protein) and a low affinity receptor for testosterone (i.e., K_d 190 nM, N_{max} 39,000 fmol/mg protein). Similarly, they found a progestin binding protein in the organism (Powell et al. 1983). As noted above, E_2 at physiological concentrations achieved during pregnancy (i.e., 10^{-8} M) stimulated endosporulation and release of endospores by the organism, which could promote pathogenesis during pregnancy (Powell et al. 1983).

16.5.6 *Rhizopus nigricans*

In the saprophytic fungus *Rhizopus nigricans*, steroids such as progesterone are toxic at high concentrations, inhibiting its growth (Jeraj et al. 2005). Steroid binding sites with high affinity for progesterone (K_d 40 ± 14 nM determined by binding, and K_d 71 ± 22 nM determined by displacement studies) and lower affinity for 21-hydroxy-progesterone or testosterone were demonstrated; no affinity was detected for 17β-estradiol, onapristone and K-naphthoflavone in the enriched plasma membrane fraction of this fungus (Lenasi et al. 2002). Further investigation into the functional response to steroid showed that progesterone induced about 30 % activation of G proteins over basal level, as determined by GTPase activity (EC50 = 32 ± 8 nM) and by the guanosine 5P-O-(3-thiotriphosphate) (GTPγS) binding rate (EC50 = 61 ± 21 nM), in the membrane fraction. The affinity of receptors for progesterone was substantially decreased in the presence of GTPγS and cholera toxin (Lenasi et al. 2002). Furthermore, the intracellular level of cAMP decreased in the presence of steroids, suggestive of a possible role for cAMP signaling in the response of *R. nigricans* to steroids. Growth analysis, in the presence of cAMP increasing agents, indicates a role for cAMP in fungal growth inhibition by steroids (Jeraj et al. 2005).

16.6 Functional Responses of Fungi and Their Genomes to Hormones

As noted in several of the studies already, when a fungus harboring one of these steroid-binding proteins is exposed to cognate hormone, direct physiological responses have been elicited. In addition to the effects on fungal growth, other physiological effects of mammalian hormones on fungal physiology have been described, such as calcium uptake (Berdicevesky and Silbermann 1982) and oxygen consumption (Shah 1981).

16.6.1 *Candida albicans*

Although E_2 blocks the dimorphic conversion of the mycelia form to the invasive yeast form in *P. brasiliensis*, in *C. albicans,* E_2 directly stimulates the dimorphic transition from yeast to the hyphal form (White and Larsen 1997; Cheng et al. 2006), suggesting that different fungi respond to the same hormone in a different way.

For *C. albicans,* genome-wide inventory of up- or down-regulated genes in the presence of E_2 and progesterone has been reported (Cheng et al. 2006; Banerjee et al. 2007). Several genes, including methionine synthase (Burt et al. 1999), *HSP90* (Burt et al. 2003), CDR1 (Krishnamurthy et al. 1998; Zhang et al. 2000; Cheng et al. 2006) and *PDR16* (Cheng et al. 2006) have been shown to be induced following E_2-treatment. Progesterone also enhanced the expression of multi-drug resistance (MDR) genes belonging to ATP Binding Cassette (*CDR1* and *CDR2*) super-family of multidrug transporters and several genes associated with hyphal induction

(Banerjee et al. 2007). Transcripts of E_2-treated *C. albicans* cells in the Cheng et al. (2006), study showed increased expression of *Candida* drug resistance genes, *CDR1* and *CDR2*, across several strain-E_2 concentration-time point combinations, suggesting that these genes are the most responsive to estrogen exposure. Expression of *CDR1* and *CDR2*, which encode multidrug transporters of the ABC family, are up-regulated in the presence of E_2 at concentrations ranging from 10^{-3} to 10^{-9} M. The drug efflux pump *CDR1* has been implicated in resistance to the azole antifungal drugs commonly used in clinical practice (Albertson et al. 1996; Sheehan et al. 1999). In a detailed study, it has been indicated that the promoter region of the *CDR1* gene contains steroid responsive elements (SRE) and a drug response element (DRE) (de Micheli et al. 2002; Karnani et al. 2004). The transcription factor *TAC1* binds to the DRE in response to E_2 and induces *CDR1* expression (Coste et al. 2004). Another AP-1 recognition element was noted upstream of the multidrug resistance gene *CDR1* in *C. albicans*, which has been shown to mediate *CDR1* induction in response to various types of drugs, including some azoles (Puri et al. 1999). The DRE consensus sequence is 5′ CGGA (A/T) ATCGGATATTTTTTTT 3′. Although this consensus sequence is found uniquely upstream of *CDR1* and *CDR2*, a more redundant form of the sequence, 5′ WCGGWWWWCGGWWW 3′ (W is A or T), is found in the promoters of *IFU5*, *RTA3*, and *HSP12* (de Micheli et al. 2002; Coste et al. 2004). Further investigation on SRE indicated that it consists of two distinct elements, viz. SRE1 and SRE2. Although SRE1 responds only to progesterone, SRE2 responded to both progesterone and E_2. Both SRE1 and SRE2 were specific for steroids, as they did not respond to drugs, such as cycloheximide, miconazole and terbinafine (Karnani et al. 2004). Analysis of *C. albicans* strain DSY654, which lacks the *CDR1* and *CDR2* coding sequences, showed a significantly decreased number of germ tube-forming cells in the presence of E_2. *PDR16* was the most highly up-regulated gene in strain DSY654 under these growth conditions (Cheng et al. 2006). It is conceivable that the components of the phospholipid and sterol metabolic pathways may interact to affect *C. albicans* germ tube formation and length. Thus, increased *CDR1* expression is an adaptation response to the environment inside a human host, aimed at enhancing the organism's tolerance for steroids or drugs used to treat fungal infection.

The neuroendocrine hormone, serotonin, has also been shown to modulate several virulence properties of *C. albicans* in *in vitro* studies (Mayr et al. 2005). The presence of serotonin (5-hydroxytryptamine) reduces hyphal elongation, phospholipase activity and production of secreted aspartyl protease. Interestingly, a serotonin reuptake inhibitor, sertraline, also had a similar effect on *C. albicans*, reducing the same virulence properties and reduced fungal viability (Lass-Florl et al. 2003).

16.6.2 Saccharomyces cerevisiae

Banerjee et al. (2004), carried out a genome-wide expression profile of human steroid progesterone response (1 mM progesterone exposure for 30 min) in *S. cerevisiae*. These studies revealed that the most highly up-regulated genes included two ABC transporters *PDR5* (11-fold), a close homolog of *CDR1* of *C. albicans*, and *SNQ2*

(fivefold), both of which are known to be involved in pleiotropic drug resistance. Three other consistently up-regulated genes were *ICT1*, *OYE2*, and *YAL061W,* which according to the *Saccharomyces* genome database (SGD), do not have a known function assigned to them. Among these, *OYE2* and *YAL061W* belong to the category of oxidoreductase/dehydrogenase genes (SGD), the homologs of which have been found to be highly over-expressed in *Candida* populations treated with inhibitory concentrations of antifungal drugs (Cowen et al. 2002). The consistently down-regulated genes *MTD1* (3.5-fold) and *ADE17* (threefold) are both involved in *de novo* purine biosynthesis.

In *S. cerevisiae*, which lacks endogenous estrogen receptor (ER), it appears that the protein essential to estrogen receptor function is conserved among eukaryotes to such an extent that introduction of human ER into this organism is sufficient for faithful reconstitution of estrogen signaling within these cells (Knoblauch and Garabedian 1999). This transgenic expression in fungi has proven to be very valuable in understanding hormone signaling and receptor function in mammalian systems. In addition to the human-ER, a number of accessory proteins are apparently required to efficiently transduce the steroid hormone signal. In the absence of E_2, the human-ER, like other steroid receptors, is complexed with Hsp90 and other molecular chaperone components, including an immunophilin, and p23. This Hsp90-based chaperone complex is thought to repress the transcriptional regulatory activities of human ER, while maintaining the receptor in a conformation that is competent for high-affinity steroid binding. However, a role for p23 in ER signal transduction has not been demonstrated. Using a mutant human ER (G400V) with decreased hormone-binding capacity as a substrate in a dosage suppression screen in yeast cells (*S. cerevisiae*), a yeast homologue of the human p23 protein (yhp23) as a positive regulator of ER function was identified. Overexpression of yhp23 in yeast cells increases ER transcriptional activation by increasing E_2 binding. Importantly, the magnitude of the effect of yhp23 on ER transcriptional activation was inversely proportional to the concentration of both ER and E_2 in the cell. Under conditions of high ER expression, ER transcriptional activity is largely independent of yhp23, whereas at low levels of ER expression, ER transcriptional activation is primarily dependent on yhp23. The same relationship holds for E_2 levels.

16.6.3 Paracoccidioides brasiliensis

Of particular interest to our laboratory over many years has been the dramatic effect that E_2 has on the inhibition of mycelial (M) to yeast (Y) transition of *P. brasiliensis*. To examine the cellular response of *P. brasiliensis* to the presence of E_2, we followed temporal protein expression during mycelial to yeast transformation using [^{35}S]-methionine incorporation and 1-D SDS-PAGE (Clemons et al. 1989a). E_2 altered protein expression in *P. brasiliensis in vitro* when shifted to the temperature which permits mycelium-to-yeast transformation, blocking the appearance of

proteins associated with transformation or yeast and maintaining a more mycelial protein profile until later time points, where *de novo* protein expression was virtually shut down (Clemons et al. 1989a). In addition, exposure of mycelial cultures to E_2 without a switch in temperature induced the uptake of labeled methionine (see Figs. 16.1 and 16.2).

In light of the demonstration of specific E_2 binding, and effects on morphological form transition and protein synthesis, we performed *in vivo* studies in a murine model of pulmonary infection to determine whether E_2 could alter the pathogenesis of *P. brasiliensis* and explain the observed epidemiologic data of resistance of females. In the initial studies we followed morphologic transformation of conidia after pulmonary instillation. Conidial transformation into yeast was not impeded in male mice, with budding yeast forms present as early as 48 h. In contrast, female mice had progressively fewer cells in the bronchoalveolar lavage fluid and no budding yeasts were observed (Aristizabal et al. 1998). In addition, no CFU were recovered from the lungs of female mice after 2–6 weeks of infection, whereas $\log_{10}2$ or more CFU were recovered from male mice (Aristizabal et al. 1998). Thus, in female mice transformation of the conidia was severely impeded, with the mice able to clear detectable infection (see Fig. 16.3). Furthermore, we found that castrated male mice reconstituted with high-doses of E_2 initially inhibited conidial transformation to yeast and subsequent proliferation. However, these mice had recurrence of disease with progression later in infection, which we speculate may have been due to the immunoregulatory effects of the high-doses of E_2 administered to those animals. In contrast, castrated females reconstituted with testosterone were unable to restrict disease (Aristizabal et al. 2002). Taken together, these studies support the hypothesis that the interaction of the organism with E_2 contributes to the resistance of females to this infection.

As noted above, epidemiologic studies indicate a predominance of disease in males, and *in vitro* and *in vivo* studies suggestive that the female hormone (17β-estradiol, E_2) regulates or inhibits M-or-conidia-to-Y transition (Shankar et al. 2011a). Furthermore, our studies of the effects of E_2 on protein expression were suggestive of transcriptional regulation (Clemons et al. 1989a). With the availability of new molecular tools in the form of an 11,000 element random-shear microarray we constructed (Monteiro et al. 2009), we profiled temporal transcript expression to understand the molecular mechanism of how E_2 inhibits M-to-Y transition.

We assessed temporal gene expression in strain Pb01 in the presence or absence of E_2 at various time points through 9 days of the M-to-Y transition, hybridizing labeled cDNA from representative transcripts to the 11,000 element random-shear genomic DNA microarray. We chose E_2-regulated genes, subjected those respective genes present in the cloning vector for DNA sequencing to identify genes and assign biological function.

E_2-treatment affected global gene expression of 550 array elements. Of these, 331 elements showed up-regulation and 219 showed down-regulation at one or more time points ($P \leq 0.001$). Heat shock genes (hsp90 and hsp70) are induced during M-to-Y transformation but showed low expression after 4 or 12 h exposure to E_2, whereas hsp40/Dnaj had higher expression. Genes in pathways involved in

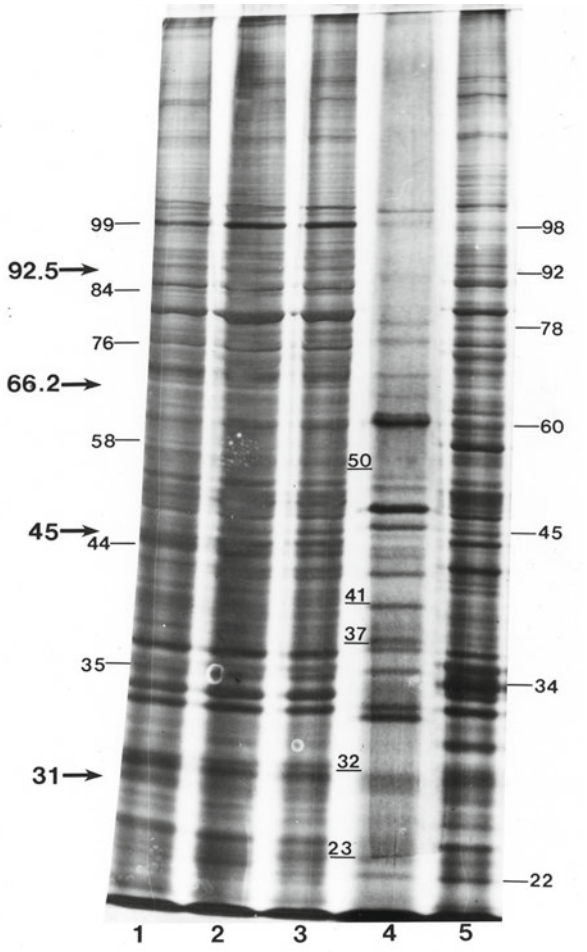

Fig. 16.1 Comparison of proteins from the cytosol fractions of E_2-treated *P. brasiliensis* undergoing M to Y transition with M and Y controls. Proteins were resolved through 9 % SDS-PAGE and silver stained. *Lane 1*, M-cells incubated solely at 25 °C, treated with E_2 for 24 h; *lanes 2–4*, M-cells after 24, 72, and 120 h of E_2 treatment at 37 °C, respectively. *Lane 5*, Y-cells incubated at 37 °C only. *Lanes 1–4* represent M cultures treated with E_2 (2.6×10^{-7} M), and *lane 5* represents the Y control. Note the maintenance of the M-form profile at 24 and 72 h (*lanes 2 and 3*) and the decreased total number of bands by 120 h (*lane 4*), which demonstrates little similarity to the Y-form profile (*lane 5*). For reference, the molecular mass in kDa of the transition bands (between *lanes 3* and *4*) as well as selected M-specific (near *left*) and Y-specific (near *right*) bands are indicated. Molecular masses in kDa of standards are indicated on the far *left*. Reprinted with permission from the Society for General Microbiology, United Kingdom from reference (Clemons et al. 1989a)

energy metabolism, as well as several retrotransposable elements also showed differential regulation in response to E_2 exposure. We (Monteiro et al. 2009), and others, have demonstrated mycelial-specific and yeast-specific genes. Interestingly, our temporal study revealed several Y-related genes, including α-1,3-glucan synthase, mannosyl transferase and Y20 that demonstrated low or delayed expres-

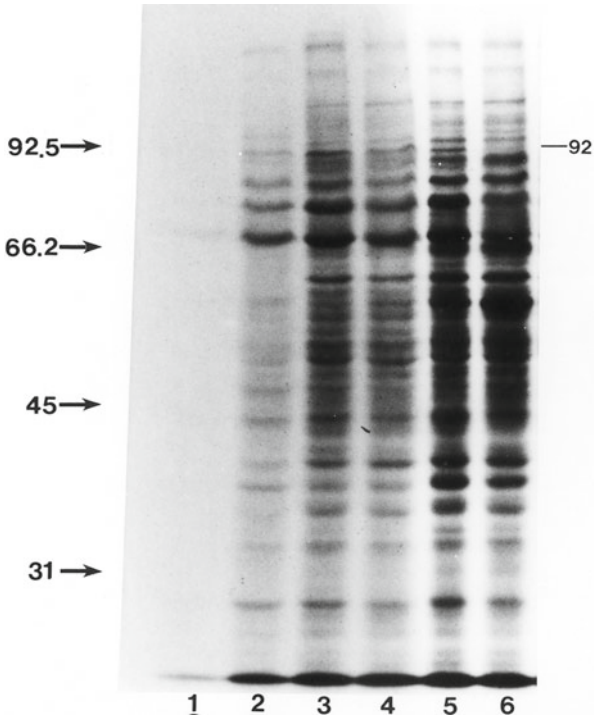

Fig. 16.2 Comparison of [^{35}S]methionine-labeled proteins in cytosol fractions of E_2-treated and untreated *P. brasiliensis* undergoing M to Y transition. Labeling was done for 2 h in the absence of radioinert methionine prior to disruption of organisms and extraction of cellular proteins. Lanes were loaded with equal counts of [^{35}S]-labeled proteins, electrophoresed and processed for fluorography. Lane assignments: *lane 1* M control, *2* M E_2,-treated, *3 and 5* M controls grown at 37 °C for 24 and 72 h, respectively. *Lanes 4 and 6* are M treated with E_2 (2.6×10^{-7} M) grown at 37 °C for 24 and 72 h, respectively. Note the effect of E_2 on label incorporation of M-form (*lane 2*) as compared to untreated control (*lane 1*). Absence of the 92 kDa Y-specific band in *lane 6* is indicated. The fluorogram was intentionally over-exposed to enhance the bands in *lane 2*. Molecular masses in kDa of standards are indicated on the *left*. Reprinted with permission from the Society for General Microbiology, United Kingdom from reference (Clemons et al. 1989a)

sion in E_2-treated cultures, whereas expression of the mycelial-specific genes, hydrophobin and β-1,3-glucan synthase, were maintained or higher in the presence of E_2. Genes potentially involved in signaling, such as palmitoyl transferase (erf2), small GTPase RhoA, phosphatidylinositol-4-kinase, and protein kinase (serine/threonine) showed low expression in the presence of E_2, whereas a gene encoding for an arrestin domain-containing protein showed high expression. Genes related to ubiquitin-mediated protein degradation, and oxidative stress response genes were up-regulated by E_2. Thus, the effect of E_2 at the molecular level on the inhibition of the M-to-Y transition is indicative that the inhibitory actions of E_2 may be working through signaling genes that regulate dimorphism (Shankar et al. 2011b).

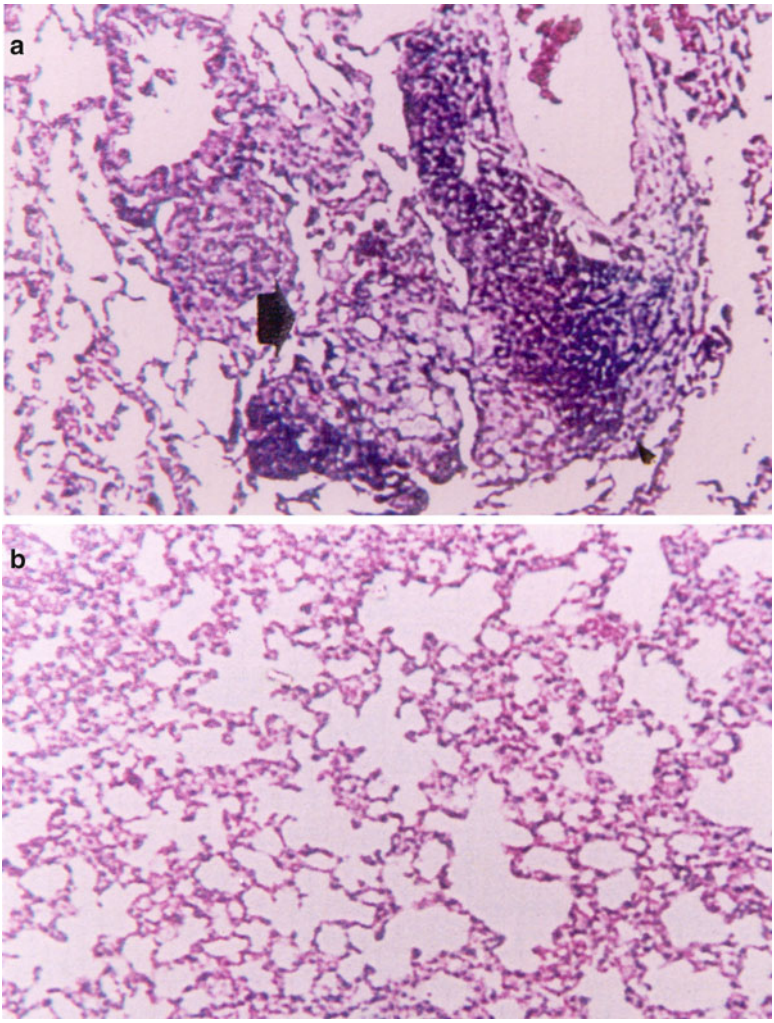

Fig. 16.3 Histopathology of the lungs in normal mice 4 weeks after infection with conidia of *P. brasiliensis* (H&E, ×100). (**a**) Normal males (NM) showing an intense chronic inflammatory reaction, also with granuloma formation and presence of yeast cells. (**b**) Normal females (NF) showing a slight inflammatory reaction, with no yeast cells present. Reprinted with permission of Oxford University Press, publisher for the International Society of Human and Animal Mycoses from the reference Aristizabal, B. H., K. V. Clemons, A. M. Cock, A. Restrepo and D. A. Stevens 2002. Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses. Med Mycol **40**(2): 169–178

16.6.4 *Aspergillus* spp.

In addition to the enhancement of growth by glucocorticoids, several species of *Aspergillus* respond to serotonin. *In vitro,* serotonin in concentrations ranging from 14 to 235 mM has antifungal properties for conidia and hyphae (Lass-Florl et al. 2002). Interestingly, serotonin receptor antagonists did not have any effect on growth (Lass-Florl et al. 2002). However, similar to data for *C. albicans,* sertaline inhibited the growth of *Aspergillus,* and the authors suggested the mechanism may be that of an interaction with a fungal transporter system (Lass-Florl et al. 2001).

16.6.5 *Cryptococcus*

Little is known about possible interactions of host hormones with species of *Cryptococcus. C. neoformans* var *grubii* and *C. neoformans* var. *neoformans* are primary etiologic agents of fungal meningitis in immunocompromised patients (e.g., AIDS), whereas *C. gattii* causes meningitis largely in immunocompetent individuals (Casadevall and Perfect 1998). Dopamine is a neuroendocrine hormone produced by the *tinea nigra* of the brain. *Cryptococcus* is able to utilize dopamine in the production of melanin, which is considered a virulence factor for this organism (Polacheck et al. 1990) and may also be related to the tropism of these fungi for the central nervous system. In recent work, the prevalence of cryptococcal meningitis in males was noted to be greater than in females. Men were more also prone to death despite having significantly higher CD4+ T lymphocyte counts. A laboratory strain and 28 clinical isolates were tested for release of capsular glucoronoxylomannan (a probable immunosuppressant and virulence factor); testosterone but not E_2 was associated with higher levels of glucoronoxylomannan release (McClelland et al. 2013).

Tamoxifen has been known to have antifungal activity (Wiseman et al. 1989). A recent study indicated that tamoxifen, which can act as an E_2 receptor antagonist, and some analogues, synergize with other antifungals against *C. neoformans in vitro* and *in vivo*, inhibit the fungus in macrophages, and appear to act by inhibition of the fungal protein, calmodulin, and related proteins (Butts et al. 2014).

16.7 Effect of Hormonal Interactions on Antifungal Therapy

We have previously discussed the interactions between hormones and drug-resistance genes in *Candida* and *Saccharomyces.* Early studies had shown ketoconazole was a competitive stereo-specific binder for the CBP in *C. albicans*, and acted as an antagonist against mammalian glucocorticoid receptors (Stover et al. 1983).

This binding did not appear related to the antifungal activity of the drug, and there appeared no corticoid effect on the activity. Others have reported inhibitory (Hogl and Raab 1980, 1982) or stimulatory (Ramondenc et al. 1998, 2001) interactions of steroids on azoles. It has been speculated that the presence of an estrogen-like 1,2 diarylethane moiety in azoles may relate to their antifungal activity, and synthesis of azole derivatives emphasizing this moiety produced compounds with antifungal activity (Massa et al. 1992).

The observation of gynecomastia in some of our patients led to our discovery that azole drugs block steroidogenesis in mammalian cells and in man, due to interference with specific P450 steroidogenic enzymes (Stevens 1985). This has led to the utility of these agents in the therapy of hypercortisolemic states and in androgen blockade in prostatic cancer.

16.8 Hormonal Effects on Pathogenesis

The direct effects of hormones on the fungi can influence the pathogenesis of the organism, such as those we described for paracoccidioidomycosis. However, there are alternative explanations for fungal pathogenesis that include hormonal effects on host mucosa, as is important in vaginal candidiasis, and possibly oral candidiasis (Junqueira et al. 2005), and hormonal effects on the host immune response (Marriott and Huet-Hudson 2006). There is an extensive literature on hormonal effects on the mucosa and on the immune response, which could profoundly affect the host-fungal pathogen interplay, and we have only cited some key articles here. Perhaps the most well-known effect of hormones on the immune response to fungi relates to the effect of glucocorticoids in promoting invasive mycoses, particularly aspergillosis and mucormycosis. The newest focus of hormone-related effects on host response to infection is on the effect of vitamin D, crucial for the development and maintenance of bones. The vitamin D precursor is created by the human skin through exposure to sunlight; more specifically, ultraviolet B rays convert stored 7-dehydrocholesterol into cholecalciferol (vitamin D_3). Vitamin D_3 is transferred via the bloodstream to the liver where it is converted to calcidiol. Vitamin D has also been shown to enhance a great variety of immune functions (Baeke et al. 2010). Of particular relevance to fungal infection, vitamin D deficiency increases Th2 responses to *A. fumigatus*, and vitamin D has been shown to down-regulate *Aspergillus*-induced Th-2 cytokines, such as IL-5 and IL-13, from cells of allergic bronchopulmonary aspergillosis (ABPA) patients; however, gliotoxin, produced by the fungus, has been shown to overcome the vitamin D/vitamin D receptor down-regulation of IL-5 and IL-13 (Kreindler et al. 2010). We were unable to show any effect of vitamin D on systemic aspergillosis in mice or its treatment (Sirivoranankul et al. 2014).

16.9 Future Directions

As is evident from the information presented in this chapter, the field of fungal endocrinology encompasses a number of different areas and shows that these simple eukaryotic organisms can indeed have true hormone receptor interactions. Additional studies are needed in the search for endogenous hormones that regulate mating of higher fungi, such as the dermatophytes, or *Coccidioides.* The influence and interactions of mammalian hormones on pathogenic fungi remains an area for study that has thus far given indications that host hormones can positively or negatively influence the pathogenesis of a fungal infection. For example, hormonal influences on *C. albicans* have been implicated in the pathogenesis of *Candida* vaginitis (Tarry et al. 2005). The question of whether these interactions occur through a fungal receptor-mediated process has begun to be addressed for *C. albicans*, where genomic expression studies have demonstrated up-regulation of genes in the organism related to drug resistance, for example. With the advent of genomics tools, such as microarrays and high throughput sequencing technologies, as well as improvements in proteomic technologies, additional studies are now possible to examine whether the hormonal influence on growth of a fungus is receptor-mediated and what cellular pathways are involved. That is to say, how does estrogen stimulate *C. albicans* or *Coccidioides* or how does progesterone inhibit the growth of *Trichophyton*?

Studies such as those we discussed will shed light on not only the hormonal interactions, but on the basic biology of the organism and the mechanisms by which morphologic change might be regulated. There remains a question of whether fungi like *C. albicans* or *P. brasiliensis* produce steroid ligands similar to mammalian estrogens. Thus, does an organism like *P. brasiliensis* make an endogenous hormone that acts as a regulator of morphology?

Lastly are the questions of what other pathogenic fungi show interactions with the host hormonal milieu and what are the responses of the organisms to the hormones? Thus, we believe that fungal endocrinology is still in its infancy as a field of research and that much remains to be done to gain a full understanding of these interactions.

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