Ming D. Li

Tobacco Smoking Addiction: Epidemiology, Genetics, Mechanisms, and Treatment

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Preface

This book, *Tobacco Smoking Addiction: Epidemiology, Genetics, Mechanisms, and Treatment*, is my attempt to provide updated knowledge and views of what we have learned about nicotine addiction from multiple disciplines. According to a recent World Health Organization report, more than 1 billion men and 250 million women currently smoke and the number of deaths caused by smoking is estimated to be over 6 million annually. Tobacco smoking is one of the most preventable causes of various cancers, especially lung cancer. How to prevent and treat tobacco addiction has become one of the most important tasks for researchers, physicians, and governments throughout the world. To reveal the susceptibility genes for nicotine addiction and successful smoking cessation, thousands of clinical and basic scientists throughout the world have been engaging in research on this behavior. Through these investigations, we have learned much about the genetics, mechanisms, pathology, and, yes, treatment of this complex disorder. Most of these accomplishments are covered in this book.

The primary reason for smokers to continue smoking is the addictive properties of nicotine, which is present in tobacco smoke. In order for nicotine to exert its pharmacologic effects, it must bind to nicotinic acetylcholine receptors (nAChRs), which are broadly distributed in almost every brain area and certain peripheral systems as well. Because of this property, research on nicotine and nAChRs has been the primary focus in the tobacco field, thus one of the primary focuses of this book.

The major mission of this book is to provide an updated knowledge, not only of the properties and biological function of nicotine and various types of nAChRs but also of the clinical aspects of tobacco smoking such as its epidemiology and treatment. To accomplish this mission, this book has been organized into 21 chapters, which can be classified into four broad sections: epidemiology, genetics, pharmacologic effects, and treatment. Briefly, Chap. 1 describes the epidemiology of tobacco smoking and its associated diseases; Chap. 2 describes the basic concepts and the techniques used to study the genetics of smoking addiction; Chap. 3 makes it clear that smoking is a heritable disease; Chap. 4 summarizes all genome-wide linkage analysis findings of various smoking behaviors; Chaps. 5, 6, 7, 8, and 9 provide updated summaries of the best-investigated candidate genes for smoking addiction, including *CHANA5/A3/B4*, *CHRNB3/A6*, GABAergic, *ANKK1/DRD2*, and the sero-

tonin system; Chap. 10 covers the converging findings from linkage and association approaches; Chap. 11 describes representative examples of the epistatic effect on smoking addiction; Chaps. 12 and 13 summarize the genes and pathways that to date have been found to be involved in addictions based on pathway and gene enrichment analyses at both the RNA and protein levels; Chap. 14 illustrates how microRNAs are involved in smoking addiction; Chaps. 15, 16 and 17 discuss how nicotine affects food intake and body weight, inflammation, the immune system, and cancer development; Chap. 18 shows how genes encoding different nAChR subunits evolved in both vertebrate and invertebrate species; Chap. 19 discusses the treatment of nicotine addiction from the psychological and genetic points of view; Chap. 20 describes the status of E-cigarettes and its developmental trend; and Chap. 21 discusses the challenges and opportunities we are facing today concerning the basic and clinical aspects of smoking addiction and other psychiatric disorders as well.

This book represents a collection of major studies that were conducted and reported since 1998 by my research team at the University of Tennessee Health Science Center, the University of Texas Health Science Center at San Antonio, the University of Virginia, and Zhejiang University with financial support primarily from the National Institute on Drug Abuse of the National Institutes of Health through various grants. During these years, numerous scientists have participated in our research projects, and I am grateful for their valuable contributions to our accomplishments in the past, especially for those who contributed to the chapters included in this book, which include Drs. Rong Cheng, Wenyan Cui, Bhagirathi Dash, Tongyuan Hu, Justin Kane, Ozlen Konu, George Lou, Yunlong Ma, Chamindi Seneviratne, Andrew van der Vaart, Ju Wang, Li Wen, Jackie Yang, and Zhongli Yang, to name a few. I thank Drs. Sulie L Chang (Seton Hall University), Robert Elston (Case Western Reserve University), Joel Gelernter (Yale University), Harold Gordon (NIDA, NIH), David Goldman (NIAAA, NIH), Bankole Johnson (University of Maryland), Caryn Lerman (University of Pennsylvania), Lanjuan Li (Zhejiang University School of Medicine), Joni Rutter (NIDA, NIH), Thomas Payne (University of Mississippi Medical Center), and Jonathan Pollock (NIDA, NIH) for their collaboration and support during these years. Furthermore, I want to thank Dr. David Bronson and Ms. Judith Gunn Bronson for their excellent editing of almost all the works published by my team and almost 30 years of friendship since my graduate school days at the University of Minnesota. Especially, I am the most grateful to my wife and colleague Professor Jennie Ma of University of Virginia and my three daughters, Maria, Sophia, and Andria, for their love and continuous support during all these years. Without their participation, collaboration, and support, it would have been impossible for me to accomplish all the tasks I have set myself. Last but not at least, I am most grateful for all these people who have taught and inspire me through their contributions and for the knowledge they will convey to all who read this book.

Charlottesville, VA, USA Ming D. Li
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August, 2017

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Chapter 1
The Prevalence of Smoking and Its Associated Diseases

Abstract Smoking is the leading risk factor for several serious diseases and causes an enormous economic burden for the individual and society. Approximately six million deaths in the world annually are attributable to smoking. Thus, it is urgent to enhance the awareness of the harm caused by smoking and to develop additional effective ways to achieve smoking cessation. In the past decades, a great number of epidemiological studies have been performed to reveal the patterns of smoking and its associated diseases. In this chapter, we briefly introduce smoking prevalence worldwide and overview the investigation of smoking-associated diseases, including cancers and psychiatric disorders.

Keywords Prevalence · Smoking · Epidemiology · Smoking-related diseases · Cancers · Tobacco dependence

1 Introduction

Cigarette smoking is a chronic and relapsing addictive trait harmful to public health. According to statistics from the World Health Organization (WHO 2013), smoking kills approximately six million people worldwide each year, with more than five million of those deaths resulting from direct cigarette smoking and more than 600,000 from secondary or passive smoke exposure. The number of smoking-related deaths is expected to increase to more than eight million annually by 2030 if the current pattern of smoking continues unabated (Eriksen et al. 2013).

The main deadly effect of smoking is a variety of severe diseases, such as cancers and psychiatric disorders. More than 25% of all cancer deaths can be attributed to smoking, especially those from lung cancer, for which about 80% are caused by tobacco smoking (CDC 2010). Moreover, multiple lines of evidence show that a large amount of the morbidity and premature deaths in schizophrenia patients can be attributed to smoking-related diseases (Brady et al. 1993; Crump et al. 2013).

Extremely high healthcare expenditures are associated with smoking-related illnesses worldwide. It is estimated that globally, more than US$500 billion in economic damage is caused annually by tobacco smoking. In the United States, the total of public and private healthcare costs related to tobacco smoking were

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estimated to be about US$170 billion each year (Ekpu and Brown 2015), and in the United Kingdom, the direct expenditures of the British National Health Service (NHS) attributable to smoking have been estimated at between £2.7 billion and £5.2 billion, about 5% of the total annual NHS budget (Allender et al. 2009; Callum et al. 2011; Ekpu and Brown 2015). Furthermore, in some developing countries, the economic damage from smoking has substantially increased in the past decade. For example, in China, about USD 6.2 billion was spent for direct smoking-attributed healthcare costs and USD 22.7 billion for indirect economic costs in 2008, the direct and indirect costs were rose by 154% and 376%, respectively, compared with the costs in 2000 (Yang et al. 2011).

Prevention of smoking initiation and promotion of smoking cessation, coupled with regulations and legislation, remain to be effective ways to control tobacco use (Koplan and Eriksen 2015; Yang et al. 2015; Zhu et al. 2012). Although abundant benefits accrue from smoking cessation, the cessation rate is still low in many countries. A variety of factors have been proposed as causes of the difficulties of obtaining and maintaining smoking cessation, including psychological, genetic, pharmacologic, and social factors (Li and Burmeister 2009). One of the most important factors is nicotine dependence (ND), which is the main contributor to the persistence of smoking (Gunby 1988). Growing evidence (Baker et al. 2007; Branstetter et al. 2015; Branstetter and Muscat 2013; Mercincavage et al. 2013) has shown that time to the first cigarette of the day, one of the best indicators of ND (Fagerstrom 2003), is associated with the likelihood of smoking relapse and with withdrawal symptoms, nicotine intake, tobacco-related carcinogen exposure, and cancer risk. Furthermore, many twin and family studies have shown consistently that the risk of ND is heritable, with an average heritability of 0.59 in male and 0.46 in female smokers (see Chap. 3 for details).

In light of the severe impact of smoking on the individual and society, many studies have examined the epidemic pattern of smoking and its associated diseases. To help control the trend to more smoking, a battery of effective systemic and scientific measures should be implemented with the hope of assisting in the implementation of current cessation methods and accommodating the specific conditions of particular countries in order to reduce the demand for tobacco. In the following sections, we briefly review the prevalence of smoking in the world and summarize the harmful influence of smoking on people's health.

2 The Global Prevalence of Smoking

There are about one billion cigarette smokers worldwide (Mackay et al. 2013), amounting to approximately 30% of men and 7% of women (Gowing et al. 2015). Smoking rates differ widely between populations across the world (Fig. 1.1). A series of factors impact the prevalence of smoking and trends in prevalence, such as individuals' educational level, national economic development, and tobacco control policies. In developed countries, such as the United States and the United Kingdom,

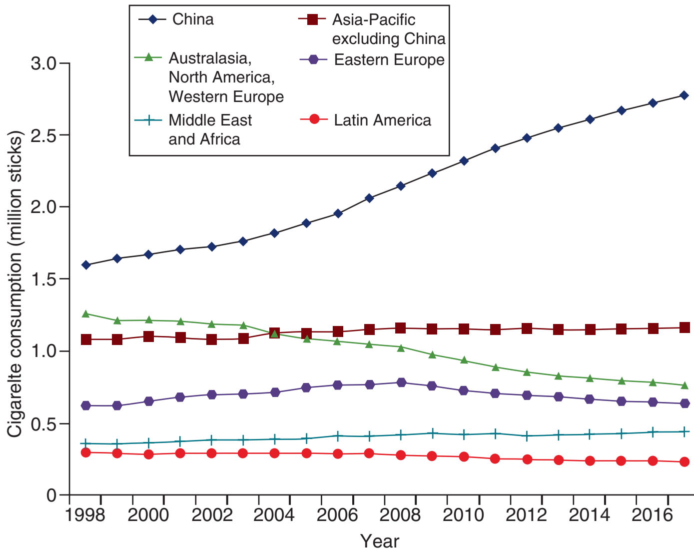

Fig. 1.1 Cigarette consumption (millions of sticks) by region (historic and forecast on retail volumes), 1998–2017 (Source: Euro monitor data; downloaded 7 May 2014)

the prevalence of smoking increased sharply in the earlier twentieth century, partly as a result of the low prices of cigarettes. The prevalence of smoking has been estimated to have been 37% among men and 25% among women. However, because of better public awareness of smoking as a hazard and the implementation of stringent legislation against smoking in the Western European countries and the United States, smoking prevalence has been greatly reduced. From 1990 to 2009, tobacco consumption in Western Europe declined by about 26% (Brathwaite et al. 2015). In the United States, the proportion of smokers declined from 20.9% in 2005 to 15.1% in 2015 (Jamal 2016).

In contrast, the prevalence of smoking has increased remarkably in low- and middle-income countries (Benowitz 2008). During the years 1990 to 2009, tobacco consumption increased by 57% in Africa and some Middle Eastern countries (Brathwaite et al. 2015). Throughout the world, more than 80% of smokers now reside in poor countries, especially in Eastern and Southeastern Asia and Africa (Stewart 2014). For example, in China, cigarette consumption in 2016 is approximately twofold higher than it was in 1998 (Gilmore et al. 2015). As the largest user of tobacco worldwide, the smoking rate in China remains high. The nation consumes more than 30% of the world's cigarettes, and two-thirds of men smoke (Chen et al. 2015; Li et al. 2011; Yang 2014). In China, many smokers do not fully understand the damaging consequences of smoking, and social conventions have linked smoking with a positive image (Yang et al. 2015; Zhang et al. 2011), which plays an important role in preventing smoking cessation.

The prevalence of smoking in men and women differs greatly in different regions of the world (Gowing et al. 2015). Globally, smoking prevalence in men is more

than four times that in women (West 2017). In developing countries, the prevalence of smoking in men is much higher than that in women. For example, there was an estimated prevalence ratio of 22 to 1 for men to women in China (Li et al. 2011). In Eastern, Southeastern, and Western Asia, the prevalence is estimated to be approximately 40% in men, whereas only approximately 4% of women smoke (West 2017). One reason for this phenomenon is that female smoking is considered socially unacceptable (Giovino et al. 2012; Jung-Choi et al. 2012). The difference is much less in most developed countries (West 2017). For example, the prevalence of tobacco smoking among women in the United States is estimated to be 13.6%, which is close to the prevalence of 16.7% among men (Jamal 2016). Moreover, the total number of male smokers in the leading three tobacco-using countries, e.g., China, India, and Indonesia, accounted for 51.4% of the world's male smokers in 2015, whereas the United States, China, and India were the leading three countries in the total number of female smokers, yet they accounted for only 27.3% of the world's female smokers (Ali and Hay 2017), suggesting that the epidemic of smoking is less geographically concentrated for women than for men.

3 Smoking-Related Cancers

Cigarette smoke contains biologically significant concentrations of known carcinogens. So far, more than 60 carcinogens have been identified in cigarette smoke (Hecht 2003), which include polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (particularly NNK and NNN), and aromatic amines; all play an important role in carcinogenesis (Pfeifer et al. 2002). Furthermore, nicotine per se not only is the main addictive component responsible for the persistence of smoking but also makes a genotoxic contribution to the etiology of cancer (Grando 2014). From the molecular point of view, most of these smoke-based carcinogens induce mutations and epigenetic reprogramming with a requirement for metabolic activation to form DNA adducts (Hecht 2003). For example, several epigenetic studies demonstrated that abnormal DNA-methylated (DNAm) loci caused by smoking are enriched in important genes and biological pathways implicated in cancers, and abnormal DNAm loci can enhance the potential for DNA damage and mutations that increase the risk of the initiation and progression of cancers (Alberg et al. 2014; Stewart 2014; Ma and Li 2017; Yang and Li 2016). For a detailed description of how methylation plays a role in the development of cancers by smoking, please see Chap. 17.

Numerous cancers have been attributed to smoking, such as cancer of the lung, mouth, lip, throat, bladder, kidney, breast, ovary, pancreas, stomach, liver, and cervix (Vineis et al. 2004). Notably, smoking is the single most important risk factor for lung cancer. In developed countries, it is estimated that 87% of all lung cancer deaths are attributable to smoking (Zon et al. 2009). A temporal change pattern of lung cancer death rates of European Americans and African-Americans from 1930 to 1996 corresponded to the wave of historical patterns of smokers (International

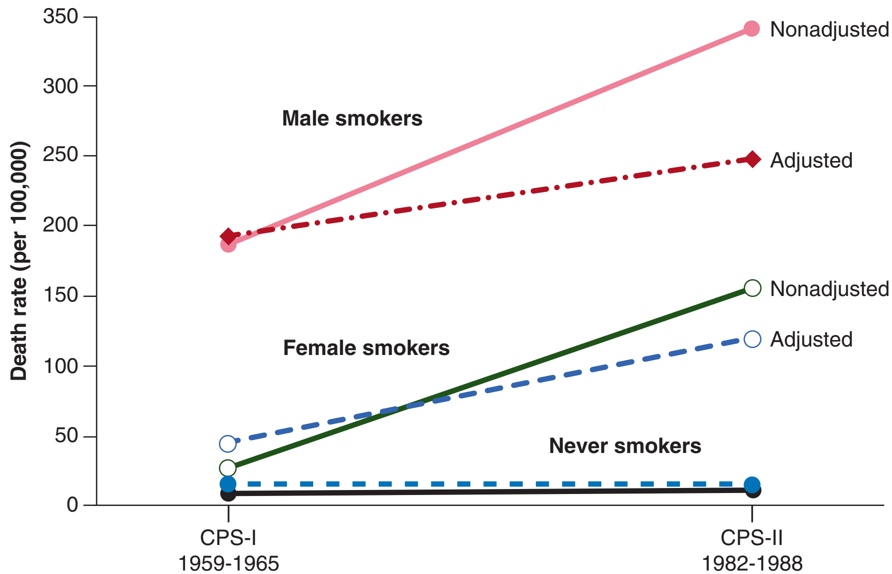

Fig. 1.2 Death rates from all lung cancers, by smoking status, Cancer Prevention Study I (CPS-I) and Cancer Prevention Study II (CPS-II), 1959–1965 and 1982–1988

Agency for Research on Cancer 2004). Based on follow-up of large populations over two decades (CPS-I, *N* = 786,387; CPS-II, *N* = 711,363) (Garfinkel and Stellman 1988; Stellman and Garfinkel 1989a, b; Thun and Heath 1997; Thun et al. 1997), the risk of death from lung cancer for non-smokers was constant across the two decades, whereas for current smokers, the risk increased dramatically (Fig. 1.2). Furthermore, the risk of cancers in smokers parallels the years of smoking and the numbers of cigarettes smoked per day (Table 1.1). For example, a 20-year follow-up study showed that those who smoked for 45 years had an increase of more than 100-fold in the annual excess incidence of lung cancer compared with 15-year smokers (Doll and Peto 1978).

Of note, the risk of cancers decreases significantly after cessation, particularly for persons who smoked for only a short time (Table 1.1). Participants who stopped at an earlier age had a lower lifelong risk of lung cancer than those who continued to smoke (Peto et al. 2000). Compared with the lung cancer death rate among current smokers, former smokers showed a progressive benefit for those who achieved cessation at earlier ages (Doll et al. 2004; Peto et al. 1992). Moreover, there is incontrovertible evidence that reduction in tobacco smoking prevents more than one-third of cancer deaths (Jemal et al. 2008). Significantly, many patients continue to smoke even after the initial diagnosis of a smoking-related cancer. For example, 23–35% of individuals with head/neck cancer and 13–20% of those with lung cancer continue to smoke after the diagnosis (Gritz et al. 2006; Nayan et al. 2011).

Table 1.1 Association between duration of cigarette smoking, amount of cigarette smoking, and years since quit before diagnosis and death as a result of breast cancer and all causes

Cigarette smoking variable before diagnosis	No. (%) of patients (*N* = 20,691)	Cause of death			
		Breast cancer		All causes	
		No. of deaths (*n* = 2894)	HR[a] (95% CI)	No. of deaths (*n* = 6778)	HR[a] (95% CI)
Duration of smoking, years					
Never smoker	10,399 (50)	1448	1 (reference)	3234	1 (reference)
Former smoker					
< 15	2376 (11)	275	0.92 (0.81–1.05)	480	0.95 (0.86–1.04)
15–30	2132 (10)	238	0.82 (0.71–0.94)	562	0.95 (0.87–1.04)
≥30	1725 (8)	242	1.10 (0.95–1.27)	868	1.39 (1.29–1.50)
Recent smoker					
<15	187 (1)	130	1.12 (0.93–1.35)	218	1.21 (1.05–1.40)
15–30	1104 (5)	180	1.10 (0.94–1.29)	379	1.49 (1.33–1.66)
≥30	2768 (13)	381	1.39 (1.23–1.56)	1037	1.92 (1.78–2.06)
Years since quit					
Never smoker	10,399 (50)	1448	1 (reference)	3234	1 (reference)
≥25	1660 (8)	175	0.93 (0.79–1.09)	459	0.97 (0.87–1.07)
15 to <25	1713 (8)	189	0.84 (0.72–0.98)	457	1.00 (0.90–1.10)
10 to <15	939 (5)	104	0.82 (0.67–1.00)	272	1.05 (0.92–1.19)
5 to <10	1122 (5)	152	0.97 (0.82–1.15)	392	1.26 (1.13–1.40)
< 5	799 (4)	135	1.20 (1.00–1.44)	330	1.52 (1.35–1.71)
Recent smoker	4059 (20)	691	1.25 (1.13–1.38)	1634	1.68 (1.58–1.80)

HR hazard ratio

[a]HR stratified by age at diagnosis, study phase, state of residence, and stage at diagnosis and adjusted for education, body mass index, age at first birth, menopausal status, use of postmenopausal hormone therapy, mammography history, alcohol consumption, and first-degree family history of breast cancer cited from a study published by Passarelli et al. [1]

4 Smoking-Related Psychiatric Disorders

Smoking behaviors are common brain disorders, which are comorbid with other psychiatric disorders, including major depressive disorder and schizophrenia. Smokers often feel that there are anxiolytic and antidepressant effects of cigarette smoking. Although the strongly popular view that smoking can decrease the negative impact of neuropsychiatric disorders continues, there are studies suggesting that the association is in the opposite direction, namely, that smoking confers risks in psychiatric diseases. For example, people are accustomed to thinking that cigarette smoking has a protective role in Alzheimer's disease, but there is an opposite case that smoking is a major risk factor for both Alzheimer's and vascular dementia (Ferri et al. 2011). Furthermore, Boden and colleagues suggested that cigarette smoking increases the risk of depression (Boden et al. 2010). So far, however, the causal direction of the association between smoking and psychiatric disorders remains largely unknown.

Here, we introduce the correlation of cigarette smoking with schizophrenia as an example of comorbidity. The smoking prevalence is substantially higher in persons with schizophrenia than in the general population (de Leon and Diaz 2005; Kelly and McCreadie 2000). In developed countries, approximately 76% of male patients with schizophrenia are smokers (Huang et al. 2016). Multiple lines of evidence indicate that the morbidity and premature deaths in individuals with schizophrenia are attributable to smoking-related illnesses (Brady et al. 1993; Crump et al. 2013). In light of the comorbidity having a harmful effect on public health, it is important to understand the biological mechanisms underlying the association between schizophrenia and cigarette smoking. There are three nonexclusive hypotheses to explain the comorbidity (Gage and Munafò 2015): (1) cigarette smoking causes the initiation of schizophrenia; (2) schizophrenia leads to the development of ND; and (3) there are shared environmental and genetic factors that predispose to both phenotypes. More genetic and psychological-based studies are warranted in the future to determine which of these hypotheses is/are correct.

5 Concluding Remarks

Cigarette smoking contributes to various diseases, which cause approximately six million deaths worldwide each year. There are approximately one billion cigarette smokers worldwide, and there exist remarkable differences in smoking prevalence between regions. In developed countries, the rate of smoking has been steady or declining, whereas in developing countries, the prevalence of smoking has increased greatly in recent years. Whereas the difference in smoking rate between men and women is much less in developed countries, the smoking prevalence in men is much higher than that in women in most developing countries. A growing number of studies have been conducted to identify the effects that contribute to smoking prevalence

as well as ND, which may help people stop smoking. Furthermore, many studies have been performed to elucidate the biological mechanism of smoking-associated cancers and psychiatric diseases. For example, lung cancer and schizophrenia are both highly correlated with cigarette smoking. Mounting evidence supports the idea that smoking cessation decreases the risk of the morbidity and death from cancers and other diseases, indicating that prevention and cessation of tobacco use is an effective way to fight smoking-related diseases. Consequently, greater efforts are needed to reduce tobacco consumption. Potentially useful measures include raising taxes on tobacco products, restraining smoking in public places (e.g., school, workplaces, and restaurants), and requiring large and graphic health warning on cigarette packages.

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Chapter 2
Addiction Genetics: Basic Concepts and Techniques

Abstract As in any other field in medicine, various technologies and analytical methods have been applied to study addictions and many other psychiatric disorders. To better understand the contents of this book and addiction genetics, this chapter presents a brief introduction to the experimental designs, types of genetic differences, molecular techniques, and statistical methods commonly used in the field. This includes family, twin, and adoption studies for the study design section and detection of point mutation, insertions and deletions, tandem repeats, variable numbers of tandem repeats (VNTRs), single nucleotide polymorphisms (SNPs), and copy number variations (CNVs) under the types of genetic differences. In the molecular technique section, various methods used to detect genetic differences are described. Regarding the statistical genetics section, both genome-wide linkage and association analysis are described.

Keywords Markers · SNPs · Linkage · Association · CNVs · Heritability · Family studies · Twin study · Mutation · PCR amplification · Gene–gene interaction · Gene–environment interaction · Point mutation · Deletion · Insertion · Microsatellite markers · RFLP

1 Introduction

Addiction genetics is a still-nascent field aspiring to imitate the tremendous development of revolutionary tools and techniques achieved elsewhere. The rapid development of new genetic methods such as high-throughput sequencing and bioinformatics technologies has greatly advanced our understanding of the pathology and etiology of most, if not all, psychiatric disorders, far beyond what we would have expected several years ago.

The primary missions of genetic research in addiction are, first, to identify the genetic factors and molecular mechanisms underlying the development of these addictive disorders and then to find ways to prevent and treat them more effectively. Such broad missions determine that molecular addiction is not a single scientific field but rather a multidisciplinary enterprise including diverse fields such as molecular biology, genetics, addiction, neurology, pharmacology, biostatistics, and

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bioinformatics. For decades, philosophers and scientists have argued about the influences of nurture (or biological inheritance) vs. nature (the environment). As our understanding of the brain has advanced, it has become clear that what really matters is the interplay between nature and nurture. To the best of our knowledge, almost all common addictive disorders such as nicotine dependence (ND) are complex disorders influenced by both genetic and environmental factors as well as by gene-by-gene and gene-by-environment interactions. There is no doubt that the field of addiction has benefited tremendously from better understanding of the role of genetics. However, genes do not equate with destiny. Not only do environmental factors impact the development of these complex addictive disorders, so does genetics. With a better understanding of brain functions, i.e., the brain's ability to shape, form, eliminate, and strengthen different neuronal networks and circuitries, we can begin to understand how brain structure and function continue to change throughout our lives. What we do in addiction genetics is to determine which genes and variants are involved and how they are expressed over different developmental stages or interact with environmental factors to shape each person's life. To become familiar with the field of addiction genetics, in the following sections of this chapter, we provide a brief introduction not only to the techniques used but also to the progress we have made with these techniques.

2 Basic Concepts and Techniques

2.1 Study Design and Heritability

Heritability is the measure of phenotypic variation that can be explained by genetic differences among the individuals who make up a population. Heritability analysis depends on the ability of the researcher to determine the relative contributions of both genetic and environmental factors to the total phenotypic variance. Both twin and adoption studies are important designs in the estimation of heritability.

Family Studies Family studies attempt to answer the question: is a disorder familial? Family-based studies typically compare the prevalence of the disorder among first-degree relatives of affected individuals (cases) with the prevalence in the population or among relatives of unaffected individuals (controls). A higher risk among the relatives of affected individuals suggests that the disorder is familial, but it does not necessarily mean that genetics is involved, as family designs cannot determine whether the causes of the similarity are genetic or environmental.

Twin Studies Twins have always fascinated the public and pose a "natural experiment" for researchers. Sir Frances Galton developed the classical twin study as early as the nineteenth century. Although Sir Galton utilized twins for the study of the role of genetics and the environment in human behavior, he was unfamiliar with the differences between monozygotic (MZ) and dizygotic (DZ) twins. The former

derive from the same egg and therefore share all of their alleles and have 100% genetic similarity, whereas DZ, or fraternal, twins derive from different eggs and are no more related to each other than are ordinary siblings.

The classical twin study exploits the shared genetic and environmental conditions of both MZ and DZ twins. In using traditional family designs, it can be difficult to separate shared genetics from the shared environment; twin studies allow these traits to be classified into genetic, shared environmental, or unique environmental components. The classical twin study remains useful when applied to estimating the contributions of genetic and environmental factors to phenotypic variance.

Although the classical twin study is the standard design for estimating heritability, there have been some modern extensions of the design to better study multiple phenotypes. In the age of behavioral genetics, it is beneficial to have extensions of the classical twin study in order to take into account multiple genes and phenotypes, especially when applied to complex behaviors. One of these extensions is the application of multivariate analysis in a statistical modeling method in which there is simultaneous analysis of correlated traits. This type of analysis involves evaluating several phenotypes along with the effects of multiple genes. Another extension is the gene expression study. These studies focus on physiological samples obtained from MZ twins. Comparison of mRNA and protein expression in MZ pairs discordant for a disease or trait of interest provides an accurate assessment of the pattern of differential gene usage. This study design is also called the co-twin control study.

Adoption Studies Adoption studies are yet another powerful tool available to addiction geneticists. Adoption studies are based on the comparison of the concordance between offspring behaviors and those of both their adoptive and biological parents. Similarities between offspring and biological parents suggest a genetic influence; similarities between offspring and adoptive parents suggest environmental influence. Although twin adoption studies are of great research interest, appropriate cases are rare.

2.2 Types of Genetic Differences

A mutation is a permanent change in the DNA sequence of an individual's genome. Even though the results often are adverse, mutations are essential to evolution, as they increase genetic variation, offering opportunities for biological improvement. Mutations can occur through either meiosis or exposure to ultraviolet radiation (sunlight) or chemicals. Several types of mutations occur, being classified by the number of base pairs involved. In this section, we introduce the types of mutations commonly found in a genome.

In a **point mutation** (single base substitution), one base is exchanged for another (e.g., A–G). Point mutation within the coding region of a gene can be classified into three types: A nonsense mutation is a base change that generates a premature stop codon – most likely rendering the encoded protein nonfunctional. In a missense

mutation, a single nucleotide is changed in a way that causes the codon to become a different amino acid, which may or may not alter the encoded protein's function. A silent mutation is a base change that alters the codon to one that encodes the same amino acid and thus causes no change in the protein. Sickle-cell anemia is caused by a substitution that changes one amino acid (missense mutation), whereas thalassemia results from a nonsense mutation.

Insertions and Deletions (indels) are mutations that either add or delete one or more nucleotides. Indels of one or two bases can cause frameshift mutations, which alter the reading frame of the codons so that the coding sequence downstream of the change can no longer be translated into a protein.

Different-size DNA fragments arise when a mutation changes the point in the sequence at which a specific endonuclease enzyme cleaves DNA, producing fragments of differing lengths. These mutations are known as restriction fragment length polymorphisms (RFLPs). Because endonuclease cleavage occurs only when a specific nucleotide is present, RFLP analysis is used to identify the presence of disease-causing mutations. In the RLFP technique, a DNA sample is digested with a restriction endonuclease into smaller fragments, which are then separated according to size by gel electrophoresis. The technique was the first DNA profiling technique used by forensic laboratories and still is one of the methods used for paternity testing. In addition, RFLP analysis was used extensively in early mapping studies and for genetic disease analysis. However, this technique has now become virtually obsolete, being replaced by high-throughput assays that can handle thousands of samples in one experiment.

Microsatellites, Simple Sequence Repeats (SSRs), or Tandem Repeats are repeating DNA sequences one to six base pairs (bp) in length. Microsatellites usually consist of 10- to 20-nucleotide stretches of simple mono-, di-, or trinucleotide tandem repeats (A_n, CA_n, GAC_n) and can be repeated many times. Microsatellites are used as molecular markers to determine paternity, in population genetic studies and in recombination mapping. Microsatellites can impact disease; for example, Huntington's disease is associated with the expansion of a CAG repeat in the *huntingtin* gene, where 37 to 95 repeats are found in affected subjects compared with 7 to 29 in healthy controls (Masuda et al. 1995).

Minisatellites or VNTRs are repeating sequences 10–50 bp in length that are slightly longer than microsatellites. One impressive example of a VNTR implicated in a human disease is *DRD4* VNTR. The *DRD4* exon 3 VNTR polymorphism, which alters gene expression, influences ADHD, personality traits, and several addiction-related phenotypes such as the urge to drink, subjective high, alcohol dependence, opioid abuse and dependence, and METH disorders. Another example is the dopamine transporter 3′-untranslated region VNTR, which has been associated with altered gene expression and linked to alterations in brain function in schizophrenia (Prata et al. 2009).

An **SNP** is a sequence variation in which a single nucleotide base (A, C, G, T) differs among members of a species. SNPs are the most frequent type of variation in the genome, accounting for as much as 90% of all human genetic variation, and occur on average every 100 to 300 bases along the 3-billion-base human genome. Thus, there are an estimated 10 to 30 million SNPs in a human genome. The majority of SNPs are biallelic, the most common being an A/G (or T/C) change, which is estimated to occur 63% of the time. A small proportion of tri-allelic SNPs have been detected as well.

CNVs are stretches of DNA ranging from 1 kilobase (kb) to 5 megabases (Mb) that are found in variable numbers in relation to a reference genome (Feuk et al. 2006). These CNVs may either be inherited or be caused by de novo mutation. They include both duplications and deletions and are the most common type of structural variations, accounting for 12% to 15% of observed human genome variation. A CNV at the alpha globin locus, in which three alpha globin genes are found, is a cause of alpha-thalassemia (Goossens et al. 1980). Recently, CNVs were found to contribute to several common neurological and psychiatric diseases (for reviews, see Merikangas et al. (2009)) such as addiction, autism, schizophrenia, epilepsy, Parkinson's disease, amyotrophic lateral sclerosis, and autosomal dominant Alzheimer's disease.

Epigenetics describes functionally relevant chemical modifications to the genome that do not involve changes in the nucleotide sequence. Examples are DNA methylation and chromatin changes via histone modifications (acetylation, phosphorylation, ubiquitination) – changes that result in differential regulation of transcription (Liu et al. 2008). Methylation is the addition of a methyl group to the five positions of cytosine and commonly occurs in a CpG dinucleotide context. Methylation is required for normal cellular development and plays a role in several key processes, including genomic imprinting, X chromosome inactivation, suppression of repetitive elements, and cancer.

Methylation may impact gene transcription in two ways. First, extensive methylation physically hinders the binding of transcription factors to the gene, thus silencing expression. Second, methylated DNA binds to proteins known as methyl-CpG-binding domain proteins (MBDs), which then recruit histone deacetylases and other chromatin-remodeling proteins that can modify histones. Histone deacetylation inactivates chromatin, resulting in silenced genes, whereas histone acetylation leads to gene expression. Several diseases, such as Rett syndrome, fragile X syndrome, myotonic dystrophy, and rare forms of Angelman syndrome and Prader–Willi syndrome, have epigenetic mechanisms (for review, see Tsankova et al. (2007)). In addition, clinical and animal studies demonstrate that several drugs exert their therapeutic effects through epigenetic mechanisms, examples being hydralazine, procainamide, methotrexate, valproic acid, methylphenidate, selective serotonin reuptake inhibitors, and antidepressants, to name a few (Csoka and Szyf 2009).

2.3 Molecular Techniques Used in Psychiatric Genetics Studies

Although numerous technologies have been developed in molecular genetic research, the approaches used in addictive genetics are mostly related to DNA. The following is a brief description of techniques commonly used in addictive genetics research.

DNA and Its Resources DNA can be obtained directly from various human and animal tissues or cultured cells. Commonly used human tissues are postmortem samples, white blood cells, and platelets. Regardless of the source, DNA is isolated by the following common steps: mechanical or chemical lysis of cellular and nuclear membranes, releasing the DNA; enzymatic destruction of other proteins and RNA in the cell lysate; and precipitation of the DNA strands. The resulting DNAs are then resuspended in a stabilization solution and stored at 4°C to −80°C until used.

DNA Amplification Amplification is the production of multiple identical copies of a DNA sequence. The most popular method is the polymerase chain reaction (PCR). These reactions are carried out with a small amount of DNA (called a template) that will amplify a targeted DNA region defined by two artificially synthesized single-stranded DNA sequences of about 20 to 30 nucleotides with complementary sequences (G = C and A = T) at either end of the target sequence (called primers). During amplification, the PCR mixture containing the target DNA, primers, DNA polymerase, and DNA nucleotides is subjected to a certain number of cycles, each consisting of denaturing, annealing, and elongation. Denaturing separates the double-stranded (ds) DNA into single-stranded (ss) DNA; annealing allows attachment of the forward and reverse primers to flank the 5′ and 3′ ends, and elongation extends the sequence along the template in the 5′ → 3′ direction by adding nucleotides. The amplified product is known as an "amplicon." Because of its versatility, the PCR method has become an indispensable initial tool in a wide range of technical applications used in molecular genetics study, such as DNA sequencing, genotyping, and mutation analyses. However, generally, a sequence >5000 bp long is difficult to amplify.

Restriction Endonuclease and RFLP Analysis As noted above, restriction endonucleases are bacterial enzymes that digest dsDNA at specific nucleotide sequences. The resulting DNA pieces are called "restriction fragments." Because a given restriction endonuclease digests the DNA only at a specific sequence, a point mutation at the potential digestion site will prevent cutting of the DNA strand. Therefore, polymorphisms at potential digestion sites of specific restriction endonucleases can be identified according to the length of the restriction fragments. This technique is called "restriction fragment length polymorphism (RFLP) analysis." In the RFLP technique, the DNA of interest is digested with a specific restriction endonuclease(s) at its optimal temperature for a certain period of time, and then the reaction mix is

electrophoresed on a gel. The restriction fragments mobilize differentially on gels according to their lengths. This differential separation of fragments enables the identification of the presence/absence of a mutation at the action site of the restriction endonuclease.

Microsatellite Detection Microsatellite markers are generally identified in vitro through PCR amplification using primers designed for the unique sequences that flank the 3′ and 5′ ends of microsatellites. The resulting products are separated and visualized by electrophoresis on agarose or polyacrylamide gels or by capillary electrophoresis; the amplicons with more repetitive nucleotide sequences are mobilized less than the ones with fewer repetitions. Thus, depending on their positions in the gel, the researcher can determine the size and number of repetitive sequences.

SNP Detection Novel SNPs can be discovered by sequencing DNA samples from a population and comparing them with a reference sequence using different bioinformatics tools to detect variations. There are two main sequencing strategies employed to identify novel SNPs: locus-specific amplification (LSA) of target gene regions and sequencing randomly selected regions in the genome. Compared with the random sequencing method, the LSA method has several disadvantages, such as limitation to regions with known sequences, requirement for the synthesis of oligonucleotide primers for each region to be sequenced, and production of diploid genotypes, requiring identification of SNPs as heterozygotes. Various LSA and random sequencing techniques are commercially available. A list of most if not all of the human SNPs identified to date can be found in the SNP database (dbSNP) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/snp), which contains a million nonredundant SNPs submitted by researchers throughout the world. One of the largest contributors to dbSNP is the International HapMap Project, which has evaluated a large set of SNPs in populations of African, Asian, and European descent. The dbSNP and other freely available resources have increasingly reduced the need for new biological searches for SNPs.

Detection of Known SNPs in Individual Samples On the basis of the number of SNPs that can be investigated simultaneously, SNP genotyping platforms are categorized as high-throughput, which can handle hundreds of millions of SNPs per sample, and low-throughput, which are utilized for genotyping a small number of SNPs per sample. SNPs can be genotyped with allele-specific hybridization, enzyme-based techniques, and DNA sequencing. An example of the allele-specific hybridization technique is the commercial high-throughput Affymetrix Human SNP GeneChip used in genome-wide association analysis for simultaneous genotyping of hundreds of millions of SNPs. The enzyme-based techniques are utilized by the aforementioned RFLP and commercially available *TaqMan* SNP genotyping assays that are widely used in small- and medium-scale disease association studies. Regardless of the technique used, a reliable SNP assay must identify a unique genomic locus, accurately discriminate between the two nucleotides of the SNP, use minimal amounts of DNA, be cost-effective, and have easy quality control measures.

2.4 Statistical Methods in Psychiatric Genetic Studies

Two approaches are common in addiction genetics to identify susceptibility loci or genes for a disease of interest. The first is linkage analysis, and the other is association analysis.

Linkage Analysis Genetic linkage is the tendency of two loci physically close to one another on the same chromosome to co-segregate within a pedigree. Various methods have been developed to evaluate linkage between a qualitative or quantitative phenotype and a panel of genetic loci. Model-based linkage analysis involves comparing the likelihood of a given family constellation under the hypothesis of a specific distance (in terms of recombination fraction) between the marker and the trait-influencing locus under a known genetic model, with the null hypothesis being no linkage between the marker and the trait-influencing locus. In contrast, model-free methods do not require the specification of a disease model and are based on a correlation between similarity in marker allele sharing and in phenotype between pairs of relatives, such as sib-pairs. Model-free methods tend to be more robust, but less powerful, than model-based methods. On the other hand, model-based methods can lead to fictitious evidence of linkage if the inheritance mode is specified incorrectly. Therefore, the method chosen for a linkage study depends on the nature of the dataset, including the type of trait, the type of families available, and knowledge of the underlying mode of inheritance. For complex traits, which usually do not exhibit a distinct pattern of Mendelian inheritance, model-free sib-pair linkage analysis is often favored because (1) it is relatively easy to recruit a large number of sib-pairs, who tend to be more closely matched for age and environment than more distant relative pairs, and (2) no assumptions are required for parameters such as mode of inheritance, penetrance, prevalence rate, or disease allele frequency.

Association Analysis Association, or linkage disequilibrium (LD), denotes the tendency for alleles at two linked genetic loci to be associated nonrandomly. Association analysis capitalizes on the accumulated recombination events across the whole population history, the number of which is far greater than that arising in pedigrees with a few generations, so this technique is more powerful than linkage analysis (Risch and Merikangas 1996). Two types of association studies are commonly described in the literature. The first is a population-based *case-control* association study, which compares allele frequencies in a set of unrelated affected individuals with those in a set of independent controls. The case and control populations should be matched with respect to ethnicity as well as other factors such as age and sex. However, spurious associations may be detected that may be attributable to population stratification, admixture, or other differences between the cases and the controls that are not accounted for. The second type is a *family-based* association study. This approach reduces the concern that population substructure may cause an association because it detects an association only in the presence of linkage, but that linkage need not be tight, i.e., there is no guarantee that there is a high level of LD, and the test statistic is defined as an association attributable to linkage. An association study can be either candidate gene-based or genome-wide.

Candidate gene-based association studies, which examine the relation of a set of genes selected on the basis of prior information about molecular functions to the phenotype of interest, have commonly been applied in genetic studies on addictions. Positional candidate genes are any genes in the region under a linkage peak. Because any candidate gene approach depends on knowledge of the biological mechanisms underlying a disease or on prior linkage findings, which might be neither reliable nor sufficient to cover all relevant genes or regions, genome-wide association (GWA) studies have recently been advocated in genetic studies of addictions. The main strength of a GWA study is that it permits an "agnostic" comparison using a high-density array of SNPs across the entire genome, obviating conjecture about which genes or regions are likely to harbor risk variants. Thus, it has tremendous potential to identify risk loci with relatively small effect size, much smaller than those that may be detected through genome-wide linkage analysis. One of the major limitations of a GWA study is lower statistical power because of the choice of a stringent threshold of genome-wide significance with correction for multiple testing.

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Chapter 3
Estimation of Genetic and Environmental Contributions to Smoking Addiction

Abstract Many studies of twins have shown that genetic and environmental factors play significant, and approximately equal, roles in the determination of smoking initiation (SI) and persistence (SP). Estimates of heritability (h^2) and shared (c^2) and unique (e^2) environmental effects display considerable variability for both SI and SP from one study to another, most likely because of differences in statistical analysis models, subject age, sex, sample size, origin of cohorts, and measurement of smoking behavior. By analyzing nine studies for SI and 12 studies for SP, we found that the parameters h^2, c^2, and e^2 for SI were (mean ± SEM) 0.37 ± 0.04, 0.49 ± 0.04, and 0.17 ± 0.03, respectively, in male adults and 0.55 ± 0.04, 0.24 ± 0.06, and 0.16 ± 0.01, respectively, in female adults. Further, the h^2, c^2, and e^2 for SP were 0.59 ± 0.02, 0.08 ± 0.04, and 0.37 ± 0.03, respectively, in male adults and 0.46 ± 0.12, 0.28 ± 0.08, and 0.24 ± 0.07, respectively, in female adults. Apparently, genetic factor plays a more significant role in SI and a less significant one in SP in female compared with male adults. A significant sex difference also was detected in a shared environmental factor for SI and SP. No significant sex difference was seen for e^2 in either phenotype. Together, these findings suggest that genetic and environmental factors contribute differently to the determination of SI and SP in male and female smokers.

Keywords Heritability · Family study · Twin study · Smoking initiation · Smoking dependence · Smoking persistence · Monozygotic twins · Dizygotic twins · Meta-analysis · Sex

1 Genetic Epidemiology of Smoking Dependence

There is considerable evidence from family, twin, and adoption studies for the operation of genetic factors in the vulnerability to addiction, among which the twin study represents a popular experimental design to investigate the relative contributions of genetic and environmental factors (also see Chap. 2). In the twin studies, we compared the agreement in the behavior of monozygotic (MZ) twins, who have the same genetic makeup, with that of dizygotic (DZ) twins, who share an average 50% of their genetic makeup. Twins are said to be "concordant" if both engage in the

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same behavior. Under certain assumptions, if a higher rate of concordant behavior is observed in MZ than in DZ twins, the behavior is considered to be significantly influenced by genetic factors.

In addition to estimating genetic liability, twin studies provide critical information about environmental contributions to the phenotype of interest, including shared and unique environments. Unlike the situation with most other complex disorders, environmental factors, such as the availability of a substance and exposure to it, clearly are necessary for the expression of any genetic vulnerability to an addiction.

It has been known for decades that addiction is a complex disorder and that genetics contributes substantially to interindividual vulnerability, with an estimated moderate-to-high heritability for most addictive disorders (Agrawal and Lynskey 2006; Goldman et al. 2005; Li et al. 2003). Numerous large twin studies have concluded that genetics contributes significantly to the risk of becoming a regular and dependent smoker (Li et al. 2003). Meta-analysis of numerous twin studies shows that both genes and environment play important roles in smoking-related behaviors (Li et al. 2003). Further, the proportion of genetic vs. environmental influences in different smoking stages differs by sex, with genetic factors appearing to have a larger role in SI than in SP in women, whereas the opposite is observed in men (Li et al. 2003).

A study of smoking behavior in adult adoptees and their biological and adoptive families also supports the finding that genetics influences smoking behavior in the same generation (Osler et al. 2001). Compared with twin studies, adoption studies can minimize the confounding influences of genetic and environmental effects on smoking behavior. However, it has become increasingly difficult to conduct adoption studies because few children are available for adoption in developed countries.

Moreover, evidence from twin studies suggests a large overlap among genetic predispositions to dependence on most substances. For example, nicotine and alcohol dependences share more than 60% of their genetic vulnerabilities (Goldman et al. 2005). Finally, it is important to remember that heritability is specific to the sample under study. Thus, genetic influences may differ in tandem with many factors, such as sex, age, education, socioeconomic status, and cultural composition (Li and Burmeister 2009).

2 Estimation of Mean h^2, c^2, and e^2 for SI

During the past few decades, at least a dozen twin studies have shown that genetic factors play a significant role in the determination of smoking behavior. As shown in Table 3.1 and Fig. 3.1, there exists considerable variability in the estimates of h^2, c^2, and e^2 for SI, with a range of 0.11–0.78, 0.00–0.59, and 0.07–0.36, respectively.

Table 3.1 A list of reported representative studies on smoking initiation in adult populations

Cohort number	Country	Sex	MZ (pairs)	DZ (pairs)	h^2	c^2	e^2	References
1	Australia	M	567	352	0.33 (0.15)	0.39 (0.14)	0.28	Heath et al. (1993)
2	United States 1	M	305	354	0.64 (0.16)	0.19 (0.15)	0.17	Heath et al. (1993)
3	United States 2	M	478	232	0.54 (0.19)	0.28 (0.18)	0.18	Heath et al. (1993)
4	United States	M	2204	1793	0.39 (0.23–0.56)	0.49 (0.32–0.64)	0.12 (0.09–0.16)	True et al. (1997)
5	Finland	M	1496	3440	0.31 (0.19–0.43)	0.58 (0.47–0.69)	0.11 (0.08–0.15)	Heath et al. (1998)
6	Australia	M	567	350	0.40 (0.04–0.76)	0.51 (0.15–0.85)	0.09 (0.03–0.17)	Heath et al. (1998)
7	Australia	M	274	206	0.49	0.31	0.21	Heath and Martin (1993)
8	Australia	M	293	146	0.11	0.53	0.36	Heath and Martin (1993)
9	United States	F	255	179	0.77	0	0.23	Edwards et al. (1995)
10	Australia	F	1232	751	0.67 (0.11)	0.15 (0.10)	0.18	Heath et al. (1993)
11	United States 1	F	459	383	0.58 (0.14)	0.26 (0.13)	0.16	Heath et al. (1993)
12	United States 2	F	1397	799	0.49 (0.10)	0.29 (0.09)	0.22	Heath et al. (1993)
13	Australia	F	570	351	0.56	0.30	0.14	Heath and Martin (1993)
14	Australia	F	663	400	0.74	0.03	0.23	Heath and Martin (1993)
15	Australia	F	1232	747	0.70 (0.46–0.92)	0.18 (0–0.41)	0.12 (0–0.17)	Heath et al. (1998)
16	Finland	F	1842	3703	0.32 (0.21–0.42)	0.59 (0.50–0.69)	0.09 (0.06–0.12)	Heath et al. (1998)
17	United States	F	497	354	0.78	0.07	0.15	Kendler et al. (1999)

In the columns of h^2, c^2, and e^2, the range given in parentheses below of each estimate is the 95% confidence interval for the estimate if reported; otherwise, a single value represents the reported SE

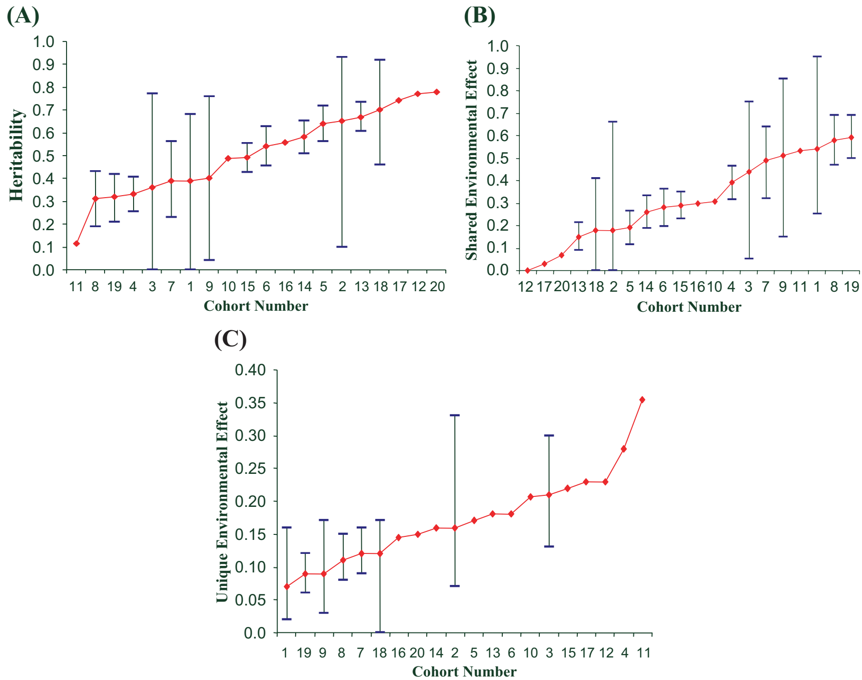

Fig. 3.1 Estimates of heritability (**a**), shared environmental effect (**b**), and unique environmental effect (**c**) of smoking initiation in different cohorts. The 95% confidence interval is given for those cohorts whose variance (or standard deviation) was reported in the original study

Table 3.2 shows the weighted mean h^2, c^2, and e^2 for male and female adults separately and for all cohorts combined using methods weighted by combined original and estimated variances, estimated variance, and cohort sample size. Regardless of which method was used, the weighted mean h^2 for female adults (0.53–0.56; $N_{cohorts} = 9$) was significantly greater than that for male adults (0.37; $N_{cohorts} = 8$; $P < 0.01$). After pooling all cohorts and collapsing on subject sex, we found that the weighted mean h^2 for SI ranged from 0.46 to 0.50. No differences were detected in the mean parameter estimates among the three weighting methods. The 95% confidence intervals for these estimates are also given in Table 3.2.

We performed meta-analytic estimates for c^2 and e^2 for male and female adults separately and for all cohorts together. As shown in Table 3.2, the weighted mean c^2 is significantly higher in male adults (i.e., 0.49 for all weighting methods) than for female adults (0.24–0.33 for all weighting methods; $P < 0.05$ or 0.01; see Table 3.2 for details). However, no significant differences were detected for e^2 among the groups of male adults (0.14–0.18) or female adults (0.15–0.16; $P > 0.05$ for all group comparisons).

Additionally, we used the paired-sample *t*-test to compare h^2 vs. c^2, h^2 vs. e^2, and c^2 vs. e^2 in male and female adults and found that h^2 is significantly greater than c^2 ($P < 0.001$) and e^2 in female adults ($P < 0.01$) but not in male adults ($P > 0.05$).

Table 3.2 Mean parameter estimates for smoking initiation in male, female, and both sex populations

Parameter/weighting method	Male adults[a] ($N = 8$)	Female adults ($N = 9$)	Total ($N = 17$)
Weighted by combined original and estimated variances	0.37 ± 0.04** (0.29–0.45)	0.55 ± 0.04 (0.47–0.64)	0.50 ± 0.04 (0.41–0.59)
Heritability (h^2)			
Weighted by estimated variance	0.37 ± 0.04** (0.29–0.45)	0.56 ± 0.04 (0.48–0.65)	0.50 ± 0.04 (0.42–0.59)
Weighted by cohort sample size	0.37 ± 0.03** (0.30–0.44)	0.53 ± 0.06 (0.42–0.64)	0.46 ± 0.04 (0.39–0.53)
Shared environmental effect (c^2)			
Weighted by combined original and estimated variances	0.49 ± 0.04** (0.42–0.57)	0.24 ± 0.06 (0.12–0.35)	0.33 ± 0.05 (0.24–0.42)
Weighted by estimated variance	0.49 ± 0.04** (0.41–0.56)	0.24 ± 0.05 (0.13–0.36)	0.33 ± 0.05 (0.24–0.42)
Weighted by cohort sample size (n_j)	0.49 ± 0.04* (0.41–0.56)	0.33 ± 0.07 (0.19–0.46)	0.40 ± 0.04 (0.32–0.48)
Unique environmental effect (e^2)			
Weighted by combined original and estimated variances	0.17 ± 0.03 (0.11–0.24)	0.16 ± 0.01 (0.13–0.19)	0.17 ± 0.02 (0.13–0.20)
Weighted by estimated variance	0.18 ± 0.03 (0.12–0.24)	0.16 ± 0.02 (0.13–0.19)	0.17 ± 0.02 (0.13–0.20)
Weighted by cohort sample size	0.14 ± 0.02 (0.10–0.19)	0.15 ± 0.02 (0.11–0.18)	0.14 ± 0.01 (0.12–0.17)

Values for all parameter estimates are given as mean ± SE; values in parentheses represent 95% confidence interval

*Significant at 5% level; **Significant at 1% level

[a]Asterisks in this column indicate a significant test (*t*-test) between male and female adults with respect to the corresponding parameter and weighting method

Parameter c^2 is significantly greater than e^2 ($P < 0.001$), again in females but not in males. These findings suggest that in women, the additive genetic effect on SI is greater than that for either the shared or the unique environmental effects. Moreover, the shared environmental effect may play a more important role than the unique environmental effect in determining SI for female but not male smokers.

3 Estimation of Mean h^2, c^2, and e^2 for SP

Similar to what was found for SI, substantial variation was observed in the reported estimates for SP parameters h^2, c^2, and e^2, with a range of 0.04–0.86, 0.00–0.57, and 0.14–0.51, respectively. For a detailed list of these representative studies, please refer to Table 3.3 and Fig. 3.2.

Table 3.3 A list of reported studies on smoking persistence or its related measures in adult populations

Cohort number	Smoking measure	Country	Sex	MZ (pairs)	DZ (pairs)	h^2	c^2	e^2	References
1	Quantity	United States	M	2390	2570	0.53[a]	–	–	Carmelli et al. (1990)
2	Persistence	United States	M	2204	1793	0.68 (0.45–0.74)	0.01 (0–0.21)	0.31 (0.26–0.38)	True et al. (1997)
3	Persistence	Finland	M	1496	3440	0.50 (0.27–0.71)	0.18 (0.01–0.35)	0.33 (0.25–0.42)	Heath et al. (1998)
4	Persistence	Australia	M	274	206	0.48	0.31	0.21	Heath et al. (1999)
5	Persistence	Australia	M	293	146	0.11	0.53	0.36	Heath et al. (1999)
6	Persistence	Australia	M	567	350	0.71 (0.31–0.84)	0 (0–0.36)	0.29 (0.16–0.45)	Heath et al. (1998)
7	Quantity	United States	M	163	166	0.52[a]	–	–	Swan et al. (1990)
8	Quantity	United States	M	2220	2373	0.49	0	0.51	Swan et al. (1997)
9	Quantity	United States	M	173	183	0.56	0	0.44	Swan et al. (1996)
10	Dependence	United States	M	1722	1484	0.60 (0.55–0.65)	0	0.40 (0.35–0.45)	True et al. (1999)
11	Regular use	Sweden	M	127	191	0.61 (0.36–0.86)	0.20 (0–0.45)	0.19 (0.02–0.36)	Kendler et al. (2000)
12	Persistence	Australia	F	1232	747	0.04 (0–0.58)	0.57 (0.07–0.72)	0.39 (0.26–0.53)	Heath et al. (1998)
13[b]	Regular use	Sweden	F	83[c]		0.64	0	0.27	Kendler et al. (2000)
14	Dependence	United States	F	497	354	0.72	0	0.28	Kendler et al. (1999)
15	Persistence	Australia	F	570	351	0.56	0.29	0.15	Heath et al. (1999)

(continued)

Table 3.3 (continued)

Cohort number	Smoking measure	Country	Sex	MZ (pairs)	DZ (pairs)	h^2	c^2	e^2	References
16	Persistence	Australia	F	663	400	0.74	0.03	0.23	Heath et al. (1999)
17	Persistence	Finland	F	1842	3703	0.49 (0.16–0.80)	0.23 (0–0.47)	0.28 (0.18–0.42)	Heath et al. (1998)

In the columns for h^2, c^2, and e^2, the range given in each parenthesis is the 95% confidence interval for the estimate

[a]Indicates unadjusted heritability estimate

[b]Parameters were estimated for women who were born from 1940 to 1958

[c]Numbers of MZ and DZ pairs were reported together in the original study

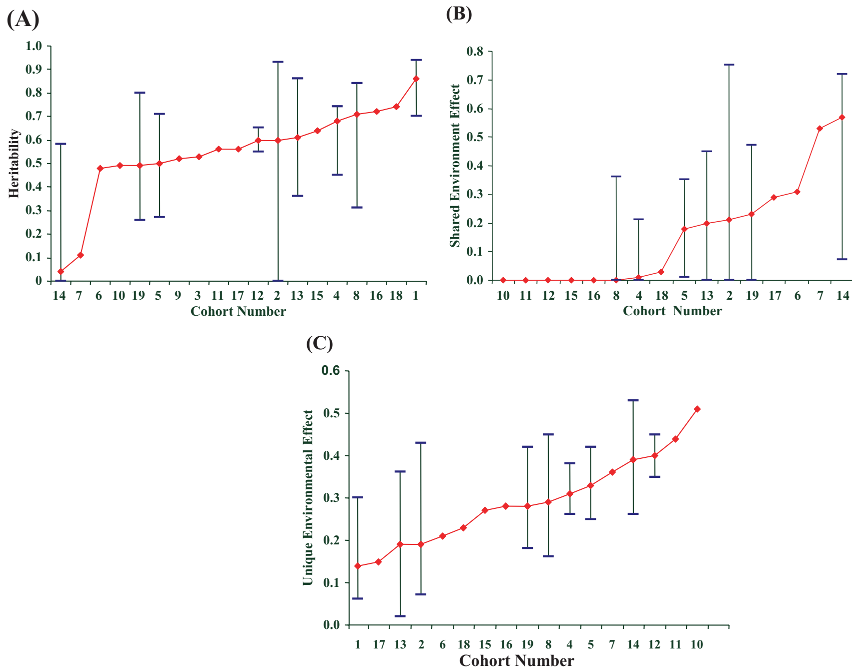

Fig. 3.2 Estimates of heritability (**a**), shared environmental effect (**b**), and unique environmental effect (**c**) of smoking persistence in different cohorts. The 95% confidence interval is given for those cohorts whose variance (or standard deviation) was reported in the original study

Similar to the approach taken for SI, the three weighting methods were employed to estimate mean h^2, c^2, and e^2 for SP in male and female adults separately and all cohorts together. As shown in Table 3.4, the weighted mean h^2 in male adults ranges from 0.55 to 0.59 (N_{cohorts} = 11), whereas in female adults, it is 0.46 (N_{cohorts} = 6). However, no statistically significant difference was detected between the groups

Table 3.4 Mean parameter estimates for tobacco dependence or its related measures in male, female, and both sex samples

Parameter/weighting method	Male adults[a]	Female adults	Total[b]
Heritability (h^2)	($N = 11$)	($N = 6$)	($N = 17$)
Weighted by combined original and estimated variances	0.59 ± 0.02 (0.54–0.63)	0.46 ± 0.12 (0.22–0.69)	0.59 ± 0.02 (0.54–0.63)
Weighted by estimated variance	0.55 ± 0.04 (0.47–0.63)	0.46 ± 0.12 (0.23–0.69)	0.52 ± 0.05 (0.42–0.62)
Weighted by cohort sample size	0.55 ± 0.03 (0.49–0.61)	0.46 ± 0.09 (0.28–0.63)	0.52 ± 0.03 (0.45–0.59)
Shared environmental effect (c^2)	($N = 9$)	($N = 6$)	($N = 15$)
Weighted by combined original and estimated variances	0.08 ± 0.04* (0–0.16)	0.28 ± 0.08 (0.12–0.45)	0.14 ± 0.04** (0.06–0.22)
Weighted by estimated variance	0.07 ± 0.04* (0–0.15)	0.26 ± 0.09 (0.09–0.43)	0.13 ± 0.04** (0.05–0.22)
Weighted by cohort sample size (n_j)	0.07 ± 0.03* (0.03–0.13)	0.26 ± 0.07 (0.12–0.40)	0.13 ± 0.04** (0.06–0.21)
Unique environmental effect (e^2)	($N = 9$)	($N = 6$)	($N = 15$)
Weighted by combined original and estimated variances	0.37 ± 0.03 (0.31–0.44)	0.24 ± 0.07 (0.11–0.38)	0.38 ± 0.02** (0.34–0.41)
Weighted by estimated variance	0.37 ± 0.03 (0.30–0.43)	0.28 ± 0.04 (0.20–0.37)	0.35 ± 0.02** (0.31–0.38)
Weighted by cohort sample size	0.38 ± 0.03* (0.32–0.44)	0.28 ± 0.03 (0.23–0.34)	0.35 ± 0.02** (0.30–0.39)

Values for all parameter estimates are given as mean ± SE; values in parentheses represent 95% confidence interval

*Significant at 5% level; **Significant at 1% level

[a]Asterisks in this column indicate a significant *t*-test between female and male adults with respect to the corresponding parameter and weighting method

[b]Asterisks in this column indicate significant *t*-test between smoking initiation and smoking persistence with respect to the corresponding parameter and weighting method

because of the large standard error in the female adult cohorts. After pooling all cohorts and collapsing on sex, our meta-analysis indicated that the weighted mean h^2 for SP ranged from 0.52 to 0.59.

We also estimated the weighted mean c^2 and e^2 for SP (see Table 3.4), which indicates that the weighted mean c^2 for SP in female adults (0.26–0.28) is significantly greater than that in male adults (0.07–0.08; $P < 0.05$ for all comparisons). Additionally, we used the paired-sample *t*-test to examine the parameters h^2 vs. c^2, h^2 vs. e^2, and c^2 vs. e^2 within male and female adults for SP independently and found that e^2 is significantly greater than c^2 in male adults but not in female adults. A significant difference also was detected in the weighted mean for h^2 and c^2 in male adults but not in female adults. However, no difference was detected in the weighted means for h^2 and e^2 in either male or female adults. These findings suggest that genetic and environmental effects play different roles in determining SP in male and female adults.

4 Differences Between Male and Female Smokers by Sex

On the basis of the combined variance method, we found that the mean heritability for SI is 48.6% (i.e., a female–male difference of 0.18) higher in female adults than in male adults, whereas the weighted mean heritability for SP in male adults is 28.3% (i.e., a male–female difference of 0.13) higher than that in female adults. These findings indicate that genetic factors contribute differently to the determination of SI and SP in male and female adults.

Using a meta-analysis of published twin studies, we found that genetic and environmental influences contribute differently to smoking behavior in males and females (see Fig. 3.3). We also found that the weighted mean c^2 for SI in male adults is approximately twofold greater (i.e., a male–female difference of 0.25) than in female adults, whereas the weighted mean c^2 for SP in female adults is almost 2.5-fold greater (a female–male difference of 0.20) than that in male adults. No significant sex differences were detected for e^2 for either phenotype. Furthermore, in male adults, the weighted mean c^2 (i.e., 0.49) of SI was much greater than that for SP (i.e., 0.08). However, the magnitude of the shared environmental effect was stable across phenotypes in women. These findings confirm the existence of significant sex differences in the contribution of genetic and shared environmental effects to the total variance in these two important smoking-related phenotypes.

Current knowledge generally supports the hypothesis of sex mediation of genetic effects. Genetic influences on smoking initiation appear to be stronger in females than in males, whereas the influences on smoking persistence are stronger in males than in females. The available longitudinal data on young smokers indicate that, compared with boys, girls have greater rates of change in the use of tobacco (Duncan

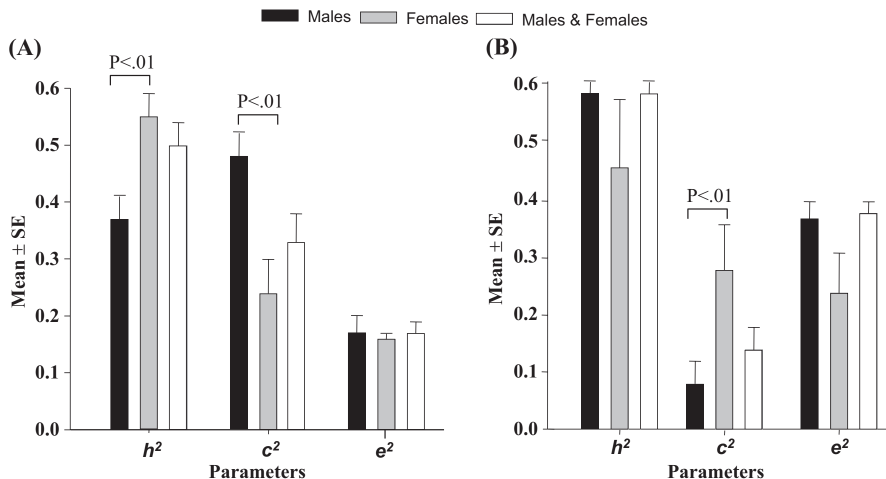

Fig. 3.3 Estimates of heritability (h^2) and shared (c^2) and unique (e^2) environmental factors among male, female, and pooled male and female smokers for smoking initiation (**a**) and smoking persistence (**b**)

and Duncan 1994), higher smoking rates (Kandel et al. 1992), higher initial use, and subsequent less rapid increase in their use of tobacco (White et al. 2002) and begin to smoke at a younger age (White et al. 2002). In a review of both human and animal studies (Perkins et al. 1999), it was concluded that sex differences may exist in nicotine effects and self-administration, and the investigators suggest that more work needs to be done to determine sex differences during the acquisition of smoking and whether the influence of sex changes across different stages of dependence. Future longitudinal research in twins will need to sort out whether the same or different genetic influences are implicated in SI and in SP and whether they differ as a function of sex. These results further suggest the need to employ a sufficient number of mixed-gender DZ pairs in twin studies so that the mediating effect of sex on parameter estimates for genetic and environmental effects can be examined directly.

5 Genetic and Environmental Contribution to SI and SP

Numerous twin studies have been reported on genetic and environmental contributions to SI and SP phenotypes. Given the inherent differences in the subject ages, sex, smoking measures, and statistical models in each study, it is not feasible to compare these estimates across different studies directly. The question then becomes how to combine estimates for genetic and environmental effects in various studies in order to estimate accurately the genetic and environmental contribution to smoking behaviors. On the basis of heritability estimates for smoking from three twin studies, in 1986, Hughes reported an arithmetic mean heritability of 0.53 with a range from 0.28 to 0.84 (Hughes 1986). However, no method for weighting by sample size or variance was used in this early paper. In 1999, an extensive review was reported by Sullivan and Kendler (1999), in which more cohorts were included, particularly for SI. The authors reported that the mean heritability was 0.56 for SI and 0.67 for SP, which are 18.0% and 13.6% higher than the corresponding values reported in our study (Li et al. 2003). In contrast, our weighted mean estimate of shared environmental effects on SI and SP are at least 37.5% greater (a difference across the two papers of 0.09 for SI and 0.12 for SP) than those reported by Sullivan and Kendler. Slight differences in the estimated unique environmental effect also are present in these two studies (i.e., a difference of 0.03 for SI and 0.07 for SP). These discrepancies may be attributable to the inclusion of several large cohorts in the present study and the use of different statistical methods. Given that more cohorts have been analyzed by three weighting methods yielding similar results in almost all instances, we believe that these parameter estimates more closely reflect the contribution of h^2, c^2, and e^2 to the total variances of these two smoking phenotypes.

6 Concluding Remarks

Through meta-analysis of twin studies on both smoking initiation and smoking dependence, we revealed that genetics plays a significant role in these two smoking-related phenotypes. More importantly, this study indicated great differences between males and females regarding the contribution of genetics to these two phenotypes. Specifically, genetic factors were found to play a more significant role in smoking initiation but a less significant role in smoking dependence, in female smokers. Significant difference by sex also was detected in shared environmental factors encouraging smoking initiation and dependence. These are highly significant findings, as they not only indicate that genetics contributes greatly to smoking initiation and dependence but also suggest that different prevention and treatment strategies are needed for men and women.

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Chapter 4
Identified Susceptibility Loci for Nicotine Addiction Based on Genome-Wide Linkage Analyses

Abstract To identify susceptibility loci for smoking dependence, more than 20 genome-wide linkage studies have been conducted in different populations using a variety of nicotine dependence (ND) assessment approaches, including smoking quantity (SQ), Heaviness of Smoking Index (HSI), Fagerström Test for Nicotine Dependence (FTND), ever smoking, habitual smoking, or maximum number of cigarettes smoked in a 24 h period (CPD). This chapter provides a critical summary of the susceptibility loci identified for ND and related measures. Although a great number of identified genomic regions did not reach the level of "suggestive" or "significant" linkage or failed to be replicated in independent studies, 14 regions, located on chromosomes 3–7, 9–11, 17, 20, and 22, have been found to be "suggestive" or "significant" linkages in at least two independent samples. Among them, the regions on chromosomes 9 (9q21.33-q33), 10, 11, and 17 have received the strongest support and deserve more attention in future genetic studies on ND.

Keywords Linkage analysis · Smoking quantity · Heaviness of Smoking Index · FTND · Suggestive linkage · Significant linkage · Susceptibility loci · Smoking dependence · Susceptibility loci

1 Introduction

As described in Chap. 3, many large-sample twin studies in the US and other countries have demonstrated that genetic factors contribute to the risk of becoming a regular smoker. Initial evidence for a genetic influence on ND came from cross-sectional twin studies that showed a mean heritability of 0.53 (range 0.28–0.84) for cigarette smoking (Hughes 1986). Our meta-analysis of 17 twin studies on genetic parameter estimates for ND showed that the weighted mean heritability for ND is 0.59 in male and 0.46 in female smokers, with an average of 0.56 for all smokers (Li et al. 2003a). Complex segregation analyses of smoking behavior in 493 three-generation families support a dominant major gene effect with residual familial correlation (Cheng et al. 2000). Together, these findings strongly suggest that ND, a complex disease, involves multiple genes and environmental risk factors, as well as interactions between genes or between genes and the environment (see Chap. 11 for

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detection of gene-by-gene interaction). Further, the proportion of genetic vs. environmental influences on different smoking stages differs by sex, with genetic factors appearing to have a larger role in smoking initiation than in ND in women, whereas the opposite is observed in men (Li et al. 2003a).

To identify susceptibility loci for ND, significant efforts have been made using an approach that tests for linkage of the disorder to polymorphic markers across the entire genome. We are aware of more than 20 published genome-wide linkage studies for smoking behavior (Bergen et al. 1999, 2003; Bierut et al. 2004; Duggirala et al. 1999; Ehlers and Wilhelmsen 2006, 2007; Gelernter et al. 2004, 2007; Goode et al. 2003; Han et al. 2010; Hardin et al. 2009; Li et al. 2003b, 2006, 2008; Loukola et al. 2008; Morley et al. 2006; Pomerleau et al. 2007; Saccone et al. 2003, 2007; Swan et al. 2006; Vink et al. 2004, 2006; Wang et al. 2005). However, only a few putative genomic linkages have been replicated in independent studies. A significant limiting factor in replicating these linkage findings is genetic heterogeneity, especially when the sample size is small or participants of various ethnic origins are included. In addition, the size of the genetic effect, the density of markers, the definition and assessment of the phenotypes, and the statistical approaches might contribute to difficulty in replicating the findings of genome-wide linkage scans. Despite these concerns and limitations, significant progress has been made, and the primary objective of this chapter is to provide an update on the progress made in identifying susceptibility loci for ND.

2 Genetic Approaches Used to Detect Susceptibility Loci for ND

Two approaches have been popular to identify susceptibility loci and genes for complex traits. The first is genome-wide linkage analysis, and the second is association analysis (see Chap. 2 for details).

“Genetic linkage” refers to the tendency of two genetic loci close to each other on the same chromosome to co-segregate within a pedigree. Various methods have been developed to evaluate linkage between a qualitative or quantitative phenotype and a panel of genetic markers, including both model-based and model-free methods. Model-based linkage analysis compares the likelihood of a given family constellation under the hypothesis of a specific distance (measured as the recombination fraction) between the marker and the trait under a known genetic model, with the null hypothesis being no linkage between the marker and the trait loci. In contrast, model-free methods do not require the specification of a disease model and are based on a correlation between similarity in marker allele sharing and in phenotype between pairs of relatives, such as sib-pairs. Model-free methods tend to be more robust but less powerful than methods based on (correct) models. On the other hand, model-based methods are less powerful and can lead to fictitious evidence of linkage if the inheritance mode is incorrectly specified. Therefore, the method(s) chosen for

a linkage study depends on the nature of the dataset, including the type of trait, the type of families available, and the knowledge of the mode of inheritance. For complex traits that usually do not exhibit a distinct pattern of Mendelian inheritance, model-free sib-pair linkage analysis is often favored because (1) it is relatively easy to recruit a large number of sib-pairs, who tend to be more closely matched for age and environment than more distant relative pairs; and (2) no assumptions are required about such parameters as mode of inheritance, penetrance, prevalence rate, or disease allele frequency. Initial linkage analysis often reveals a broad peak for a low-resolution chromosomal region linked to the trait of interest. High-resolution fine-scale mapping using additional genetic markers or association approaches usually is required to pinpoint the precise location of the causal variant.

3 Smoking Measures Used in Linkage Studies

The methods of assessing ND differ greatly from study to study. The ND measures used in reported studies have included habitual smoking, regular and persistent tobacco use, SQ, maximum number of cigarettes smoked in a 24 h period (MaxCigs24), the HSI, the Fagerström Tolerance Questionnaire (FTQ), the FTND, and DSM-IV or DSM-IV-like criteria.

4 Nominated “Significant” or “Suggestive” Susceptibility Loci for ND

So far, more than 20 linkage scans for various ND-related behaviors have been reported, most since 2005. Figure 4.1 provides a graphic summary of most of the regions that have been nominated for “suggestive” or “significant” linkage to ND. To ensure the comparability of these loci across studies, the map position of each marker or marker pair that defines the linkage region in the original study was checked against the most recent version of the human linkage map through the website www.ncbi.nlm.nih.gov/mapview/static/humansearch.html#marsh.

Table 4.1 shows eight “significant” genomic regions for ND-related phenotypes nominated in reported studies. Of these loci, the regions on chromosomes 1 and 5 were detected with the empirically genome-wide significance determined by permutation analysis of at least 1000 simulated genome-wide scans (Gelernter et al. 2007; Wang et al. 2005). The other six regions were detected with conventional one-round linkage analysis according to the theoretical threshold (Li et al. 2003b, 2006, 2008; Saccone et al. 2007; Swan et al. 2006). Unlike the regions on chromosomes 1, 12, and 16, the regions on chromosomes 5, 10, 11, 20, and 22 have been replicated by independent studies, although the logarithmic base_{10} of the odds (LOD) score or *P* value from other studies did not reach the threshold for “significant” linkage.

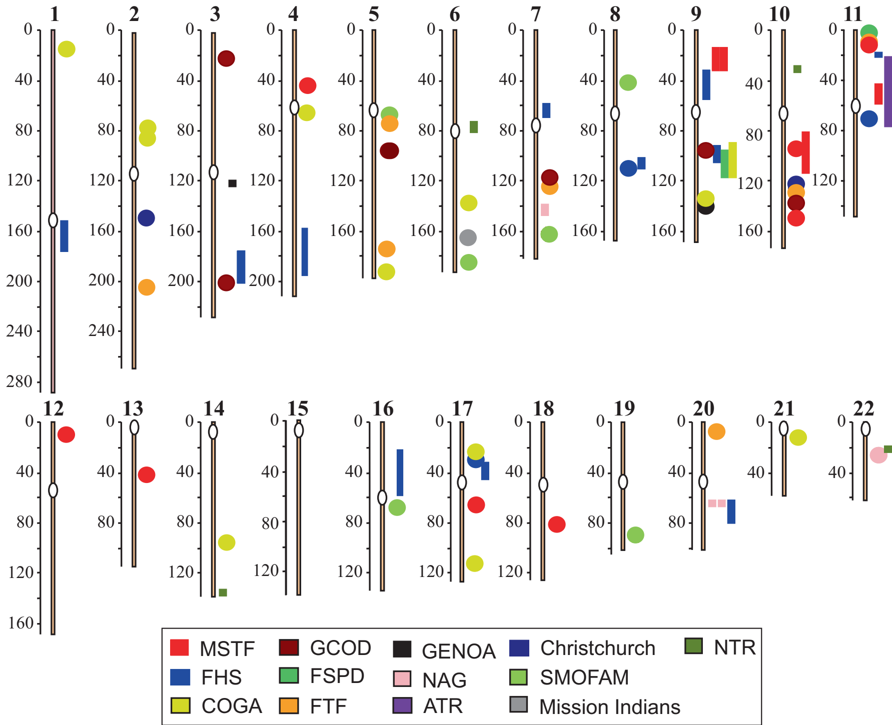

Fig. 4.1 Chromosomal locations of peaks or intervals with "significant" or "suggestive" linkage to all ND-related measures in individual or repeated analyses of data from the Collaborative Studies on the Genetics of Alcoholism (COGA), the Framingham Heart Study (FHS), the Mid-South Tobacco Family (MSTF) study, the Nicotine Addiction Genetics (NAG) project, the Finnish Twin Families (FTF), the Mission Indians in Southwest California, the Genetic Epidemiology Network of Arteriopathy (GENOA) study, the Smoking in Families Study (SMOFAM), the Netherlands Twin Register (NTR) study, the Genetics of Cocaine or Opioid Dependence (GCOD) study, the Christchurch sample of New Zealand, the Australian Twin Registry (ATR), and the Family Study of Panic Disorder (FSPD)

Interestingly, although the significant region from 151.9 to 175.6 cM (based on the Marshfield map) on chromosome 1 has received only limited support from two independent human studies (Bergen et al. 1999; Goode et al. 2003), it receives strong support from a linkage study for oral nicotine consumption in C57BL/6J × C3H/HeJ F_2 intercross mice (Li et al. 2007b). Among the four detected significant quantitative trait loci (QTLs), the locus with the largest LOD score, 15.7, was located around 96 cM on chromosome 1 (Li et al. 2007b). This region of the mouse genome is syntenic with human chromosome 1 at around 169 cM. As for the "significant" linkage for ND on chromosomes 12 and 22, it has been detected only in the combined African-American (AA) and European-American (EA) samples of the Mid-South Tobacco Family cohort (Li et al. 2008) and in the combined Australian and Finnish samples of Nicotine Addiction Genetics (Saccone et al.

Table 4.1 Nominated chromosomal regions with “significant” linkage to smoking behavior in one sample

				Max			Minimum		
Chrom	Sample	Peak or peak interval (cM)	Marker or marker region	LOD	Position	Chr. bands	P value	Smoking phenotype	Reference
1	FHS	151.9–175.6	D1S534-D1S1677		119,578,203–163,660,041	1p12–q23.3	Genome-wide $P = 0.001$	SQ	Wang et al. (2005)
5	AA/GCOD	95.4	D5S428	3.04	85,310,624–85,510,963	5q14.3	Genome-wide $P = 0.037$	FTND	Gelernter et al. (2007)
10	AA/MSTF	93.9	D10S1432	4.17	74,559,213–74,759,591	10q22.1		SQ	Li et al. (2006)
11	FHS	58.4–76.1	D11S1985–D11S2371		58,396,239–73,605,374	11q12.1–q13.4	0.000001	SQ	Li et al. (2003b)
12	Combined AA and EA samples of MSTF	6.4–26.2	D12S372–D12S391	4.44	3,487,133–12,550,226	12p13.2–p13.32		SQ	Li et al. (2007a)
16	SMOFAM	67.6	D16S145	4.0				Short-term quit: (ever quit smoking for >1 month but <1 year)	Swan et al. (2006)
20	Finnish/NAG	61.8–66.2	D20S119–D20S178	4.22	43,548,850–46,652,337	20q13.12–q13.13		MaxCigs24	Saccone et al. (2007)
22	Combined Australian and Finnish samples of NAG	21.5–27.5	D22S315–D22S1144	5.21	25,915,840–27,783,302	22q12.1		MaxCigs24	Saccone et al. (2007)

2007). Given that plausible candidate, genes with known biological functions in the etiology of dependence on nicotine and other substances of abuse are located within these regions, including ionotropic N-methyl D-aspartate glutamate receptor (*NMDA*) subunit 2B, neurotrophin 3, GABA-A receptor-associated protein-like protein 1 on chromosome 12, and β-adrenergic receptor kinase 2 on chromosome 22; more linkage and position-based association studies are greatly needed to validate these linkage results.

5 "Significant" or "Suggestive" Susceptibility Loci for ND Found in at Least Two Independent Studies

Considering that (1) numerous genomic regions have been linked to various smoking phenotypes and (2) many of these results have not been replicated in independent studies, we focus primarily on those regions that show "suggestive linkage" in at least two independent samples or "significant" linkage in one study according to the rigorous criteria proposed by Lander and Kruglyak (1995), which define an LOD of >3.6 or a P value of $<2.2 \times 10^{-5}$ as a "significant" linkage and an LOD of >2.2 but <3.6 or a P value of 7.4×10^{-4} as a "suggestive" linkage. For those reports in which genome-wide empirical P values were provided using the permutation approach, "significant" linkage was declared if the genome-wide P value was ≤ 0.05 and "highly significant" linkage if the P value was ≤ 0.001.

Under such criteria, 14 linkage regions on 11 chromosomes have been identified. These regions are summarized in Table 4.2 and Fig. 4.2. On inspection, several features become evident. First, except for chromosomes 5 and 9, for each of which two regions have been identified (regions 1 and 2), only one region was detected. Second, the regions on chromosomes 9 (from 90.3 to 127.9 cM in the Marshfield map), 10, 11, and 17 have received greater independent replication than the other regions. For example, the linkage region from 90.3 to 127.9 cM on chromosome 9 has been detected in four independent samples, namely, the Framingham Heart Study (FHS) (Li et al. 2003b), the Collaborative Studies on the Genetics of Alcoholism (COGA) (Bergen et al. 1999), the EA sample of the Genetics of Cocaine or Opioid Dependence (GCOD) (Gelernter et al. 2007), and the AA sample of the MSTF (Li et al. 2006). Within this linkage region, three genes, namely, γ-aminobutyric acid type B (*GABA*$_B$) receptor subunit 2 (*BABAB2*), neurotrophic tyrosine kinase receptor 2 (*NTRK2*), and Src homology 2 domain-containing transforming protein C3 (*SHC3*), have been identified using family-based association analysis and demonstrated to be significantly associated with ND in the MSTF sample (Beuten et al. 2005, 2007b; Li et al. 2007a). Also, the genomic region from 62 to 158 cM on chromosome 10 has been linked to ND in 5 independent populations: the Christchurch sample of New Zealand (Straub et al. 1999; Sullivan et al. 2004), the Finnish Twin Families (FTF) (Loukola et al. 2008), the EA sample of GCOD (Gelernter et al. 2007), the AA sample of MSTF (Li et al. 2006), and the EA

Table 4.2 Nominated linkage regions for ND and its related measures according to genome-wide linkage studies

Chromosome	Marker or marker region	Position	Chr. bands	Phenotype
3	D3S1763–D3S1262	167,139,681–186,323,727	3q26.1–q27.3	DSM-IV ND, SQ
4	D4S403–D4S2632, D4S244	13,650,828–65,591,728	4p15.33–q13.1	FTND, CPD
5 (region 1)	D5S1969, D5S647, D5S428	53,142,832–85,510,963	5q11.2–q14.3	SQ, smoking status, FTND
5 (region 2)	D5S400, D5S1354	168,342,870–179,731,902	5q34–q35.3	FTND, CPD
6	D6S1009, D6S1581–D6S281, D6S446	137,202,085–170,652,657	6q23.3–q27	Smoking status, FTND, withdrawal severity
7	D7S486, D7S636	115,794,675–150,799,599	7q31.2–q36.1	FTND, DSM-IV
9 (region 1)	D9S2169–D9S925, D9S925–D9S319	5,100,390–29,660,115	9p21.1–p24.1	FTND, HSI, SQ
9 (region 2)	D9S257–D9S910, D9S283, D9S64, D9S1825	90,190,735–127,988,281	9q21.33–q33.3	SQ, FTND, smoking status
10	D10S1432, D10S2469/CYP17, D10S597, D10S1652–D10S1693, D10S129–D10S217	74,559,213–129,640,525	10q22.1–q26.2	SQ, FTND, smoking status
11	D11S4046, D11S4181, D11S2362–D11S1981, D11S1999–D11S1981, D11S2368–D11S2371, D11S1392–D11S1344, D11S1985–D11S2371	1,863,635–73,605,374	11p15.5–q13.4	FTND, SQ
17 (region 1)	GATA193, D17S974–D17S2196, D17S799–D17S2196, D17S799–D17S1290	10,418,666–56,431,730	17p13.1–q22	CPD, SQ, HSI
17 (region 2)	D17S968	72,504,312–72,704,559	17q25.1	Smoking status
20	D20S119–D20S178, D20S481–D20S480	43,548,850–51,957,523	20q13.12–q13.2	CPD, SQ
22	D22S345–D22S315, D22S315–D22S1144	24,388,587–27,783,302	22q11.23–q12.1	CPD, age at first cigarette

Notes: Genomic positions for microsatellite markers and corresponding chromosome bands were obtained through the UCSC Genome Browser (http://genome.ucsc.edu/), which are in the GRCh37/hg19 assembly

Chr chromosome, *CPD* cigarettes smoked per day, *DSM* Diagnostic and Statistical Manual (American Psychiatric Association), *FTND* Fagerström Test for Nicotine Dependence, *HSI* Heaviness of Smoking Index, *SQ* smoking quantity

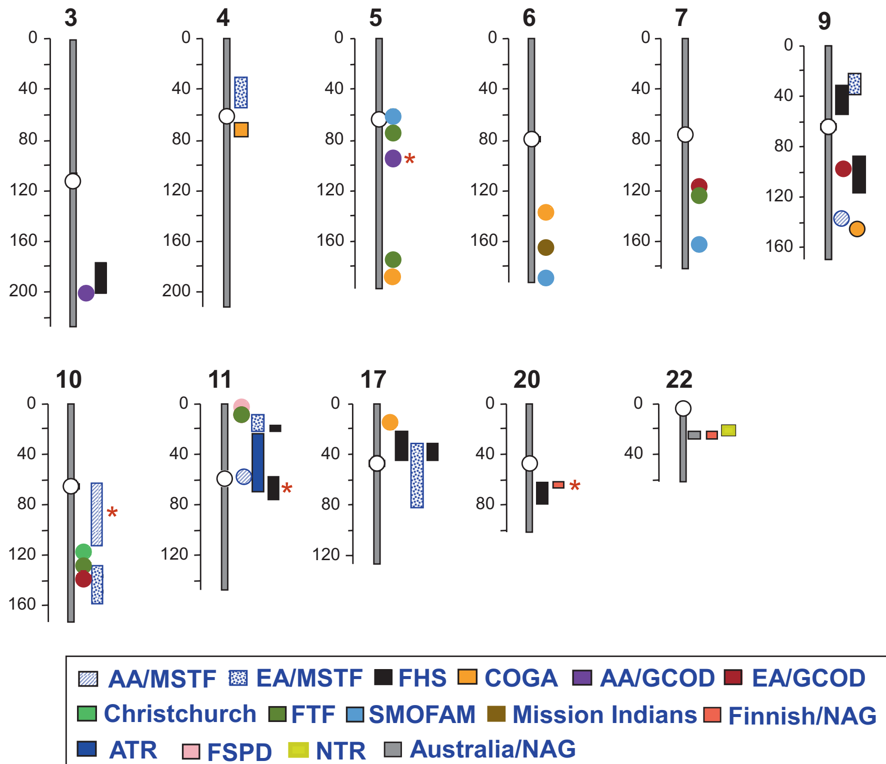

Fig. 4.2 Chromosomal locations of nominated regions for all ND-related measures with a "significant" or "suggestive" linkage score in at least two independent studies. Only chromosomes with positive linkages are shown. The linkage results were obtained from the following studies: AA/MSTF (Li et al. 2006), EA/MSTF (Li et al. 2008), FHS (Li et al. 2003b; Wang et al. 2005), AA/GCOD and EA/GCOD (Gelernter et al. 2007), COGA (Bergen et al. 1999; Bierut et al. 2004; Duggirala et al. 1999), SMOFAM (Swan et al. 2006), FTF (Loukola et al. 2008), Mission Indians (Ehlers and Wilhelmsen 2007), FSPD (Gelernter et al. 2004), Christchurch (Straub et al. 1999; Sullivan et al. 2004), ATR (Morley et al. 2006), and Finnish/NAG and Australia/NAG (Saccone et al. 2007) Abbreviations: *AA/MSTF* African-American (AA) sample of the Mid-South Tobacco Family study, *EA/MASTF* European-American (EA) sample of the Mid-South Tobacco Family study, *FHS* Framingham Heart Study, *GOCA* Collaborative Studies on the Genetics of Alcoholism, *Australia/NAG* the Australia family sample of the Nicotine Addiction Genetics (NAG) project, *Finnish/NAG* the Finnish family sample of the Nicotine Addiction Genetics (NAG) project, *FTF* Finnish Twin Families, *AA/GCOD* AA sample of Genetics of Cocaine or Opioid Dependence study, *EA/GHCOD* EA sample of Genetics of Cocaine or Opioid Dependence study, *Mission Indians* Mission Indians in Southwest California, *SMOFAM* Smoking in Families Study, *NTR* Netherlands Twin Register (NTR) study, *Christchurch* Christchurch sample of New Zealand, *ATR* Australian Twin Registry (ATR), and *FSPD* Family Study of Panic Disorder. Linkage peak marked with * on chromosomes 5, 10, 11, and 20 indicates a "suggestive linkage," as reported in the original study

sample of MSTF (Li et al. 2008). Further, the region on chromosome 11 was detected by my research group in the FHS sample (Li et al. 2003b; Wang et al. 2005) and in both the African-American (AA) (Li et al. 2006) and European-American (EA) (Li et al. 2008) samples of the MSTF cohort, as well as by Gelernter et al. (2004) in the Family Study of Panic Disorder (FSPD) sample, by Loukola et al. (2008) in the FTF sample, and by Morley et al. (2006) in the Australian Twin Registry (ATR) sample. Because β-arrestin 1 is located in this region and is an important regulator of signal transduction mediated by opioid receptors through promotion of receptor desensitization and internalization (Bradaia et al. 2005; Cen et al. 2001; Gainetdinov et al. 2004), we were motivated to determine whether the β-arrestins 1 and 2 (located in a region linked to ND on chromosome 17; see below for details) are associated with ND. Our results indicated that these two genes are significantly associated with ND in European smokers (Sun et al. 2008). Furthermore, we found the strength of these associations to be higher after removal of the SQ component from the HSI and FTND scores in both the AA and EA samples, suggesting that these two genes play a critical role in biological processes involved in the regulation of smoking urgency (Sun et al. 2008).

The region from 10.5 to 56.3 cM on chromosome 17 has been linked to ND in four studies of three independent samples, FHS (Li et al. 2003b; Wang et al. 2005), COGA (Duggirala et al. 1999), and the EA sample of the MSTF (Li et al. 2008). Because the identification of linkage of the region on chromosome 17 to ND in our genome-wide linkage scan for SQ in the FHS sample, we have conducted candidate gene-based association analyses of this region, as we did for the linked region on chromosomes 9 and 11. Our family-based association analysis revealed that GABA-A receptor-associated protein (*GABARAP*) (Lou et al. 2007), Discs, large homolog 4 (*DLG4*) or postsynaptic density protein-95 (Lou et al. 2007), protein phosphatase regulatory subunit B1 (*PPP1R1B*) or dopamine- and cAMP-regulated phosphoprotein 32-kD, DARPP32 (Beuten et al. 2007a), and β-arrestin 2 (Sun et al. 2008) are significantly associated with ND in at least one of the two MSTF samples.

Third, of the 14 nominated loci listed in Table 4.2 and Fig. 4.2, 4 showed evidence of “significant” linkage to ND. They are located on chromosome 5 with a genome-wide *P* value of 0.037 for FTND in the AA sample of GCOD (Gelernter et al. 2007), 10 with a maximum LOD score of 4.17 for SQ in the AA sample of MSTF (Li et al. 2006), 11 with a pointwise *P* value of 0.000001 for SQ in FHS (Li et al. 2003b), and 20 with a maximum LOD score of 4.22 for MaxCigs24 in the Finnish family sample of NAG (Saccone et al. 2007). Finally, although 14 susceptibility loci on 11 chromosomes are nominated here, we should not assume the regions identified in different populations are same set of genes or genetic variants. Rather, although these regions are more likely to harbor susceptibility loci for ND, the nature of the genetic variants may differ across samples.

6 Concluding Remarks

Despite inherent difficulties in conducting genetic studies on complex traits, significant progress has been made in the search for susceptibility loci for ND. By applying the same rigorous criteria for determination of "significant" or "suggestive" linkage to all reported linkage peaks for ND-related phenotypes and requiring evidence from at least two independent studies, 14 regions on 11 chromosomes have been identified. Of these, the regions on chromosomes 9 (between 90.3 and 127.9 cM), 10, 11, and 17 have been detected by the greatest number of independent studies. In addition, a list of eight "significant" linkages on chromosomes 1, 5, 10, 11, 12, 16, 20, and 22 is provided. Considering that these regions have received the most support, it is suggested they be afforded the highest priority in searching for vulnerability genes for ND in future studies.

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Chapter 5
Involvement of Variants in Gene Clusters *CHRNA5/A3/B4* on Chromosome 15 to Smoking Behaviors and Lung Cancer

Abstract Nicotine exerts its physiological and pharmacological roles in the brain through neuronal nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels consisting of five membrane-spanning subunits that affect the release of neurotransmitters such as dopamine, glutamate, and γ-aminobutyric acid (GABA) and mediate fast signal transmission at synapses. Several genome-wide association studies (GWAS) and candidate gene-based association studies investigating the genetic variants associated with ND and smoking-related phenotypes have shed light on the *CHRNA5/A3/B4* gene cluster on chromosome 15, which encodes the α5, α3, and β4 nAChR subunits. These studies demonstrate two groups of risk variants in this region. The first is marked by SNP rs16969968 in exon 5 of *CHRNA5*, which changes an aspartic acid residue to asparagine at position 398 (D398N) of the α5 subunit protein sequence and its tightly linked SNP rs1051730 in *CHRNA3*. The second one is SNP rs578776 in the 3′-untranslated region (UTR) of *CHRNA3*, which has a low correlation with rs16969968. Although the detailed molecular mechanisms underlying these associations remain to be further elucidated, recent findings have shown that α5* (where * indicates the inclusion of additional subunits) nAChRs located in the medial habenulo-interpeduncular nucleus (mHb-IPN) are involved in the control of nicotine self-administration in rodents. Disruption of α5* nAChR signaling diminishes the aversive effects of nicotine on the mHb-IPN pathway and thereby permits more nicotine consumption. This chapter provides the most up-to-date view of the progress of studies focusing on the *CHRNA5/A3/B4* gene cluster and its role in ND.

Keywords Nicotinic acetylcholine receptors · GWAS · *CHRNA5/A3/B4* · SNPrs1051730 · rs16969968 · Knockin · Knockout · Association · Lung cancer · Rare variants · Functional SNPs

1 Introduction

There are approximately 4000 compounds in cigarette smoke; however, nicotine is the primary component responsible for the development of dependence (nicotine dependence; ND). Nicotine exerts its primary role in the brain through neuronal nicotinic acetylcholine receptors (nAChRs), which are widely distributed in both

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the central and the peripheral nervous system. The nAChRs are ligand-gated ion channels consisting of five membrane-spanning subunits that can modulate the release of neurotransmitters such as dopamine, GABA, and glutamate and mediate fast signal transmission at synapses. There are 12 neuronal acetylcholine receptor subunits, with 9 α subunits (α2–α10) and 3 β subunits (β2–β4) (Elgoyhen et al. 1994, 2001; Le Novere et al. 2002). These subunits arrange in numerous distinct pentameric nAChRs, resulting in receptors that differ in distribution throughout the body and in biologic functions and other pharmacologic properties (Sargent 1993). Binding of nicotine to nAChRs creates the molecular basis for the reward provided by nicotine and, eventually, the development of ND. Thus, nAChRs represent not only plausible candidate risk factors for ND but also targets for smoking cessation efforts and personalized medicine for treating ND and other psychiatric disorders.

As shown in Chap. 3, abundant data from twin studies demonstrate that, along with environmental factors, genetic variations are responsible for ND, with an estimated heritability of about 50%. To identify susceptibility loci and genetic variants for ND and its related phenotypes, many studies have been conducted using various approaches such as genome-wide linkage analysis, candidate gene-based association, and GWAS. Of the identified genetic variants for ND, the variants in the *CHRNA5/A3/B4* gene cluster on chromosome 15, which encodes the α5, α3, and β4 subunits (Bierut et al. 2008; Saccone et al. 2007; Stevens et al. 2008; Weiss et al. 2008), have received much attention recently. Importantly, the variants in this gene cluster have been associated, not only with ND but with lung cancer (Amos et al. 2008; Hung et al. 2008; Liu et al. 2008). As a result of this genetic research, new effort has been expended to understand how variants in this region impact ND and its related phenotypes at the molecular level.

Replication of genetic association between the variants in the *CHRNA5/A3/B4* gene cluster and ND increases the validity of these findings. At the same time, it stimulates interest in exploring the molecular mechanisms of variants within this gene cluster that underlie ND. Of the significant variants in this cluster, SNP rs16969968 appears to be the most attractive as an ND factor, as it results in an amino acid change from aspartate to asparagine at position 398 of the nicotinic receptor α5 subunit protein sequence. Although the way the clustered nAChR subunits function in the development of ND is unclear, evidence from mouse models with knockout (KO) or mutations of nAChR subunits, especially the α5 subunit, suggests that disruption of α5* nAChR signaling diminishes the stimulatory effects of nicotine on the mHb-IPN pathway and thereby permits consumption of greater quantities of nicotine (Fowler et al. 2011). Hence, it is thought that variants in the *CHRNA5/A3/B4* cluster play an important role in ND through the aversive effect of nicotine on the mHb-IPN pathway, whereas there are few reports concerning the reinforcing effect of nicotine in ventral tegmental area (VTA) DA neurons (Morel et al. 2013).

To gain a better understanding of the genetic factors that contribute to ND and other smoking-related phenotypes, in this chapter, we first focus on the significant association between the variants detected in the *CHRNA5/A3/B4* gene cluster and smoking-related phenotypes and then present mechanisms that could explain such associations at the molecular level.

2 Association Between Common Variants in the *CHRNA5/A3/B4* Gene Cluster and ND

Nicotine dependence, as well as addiction to any other substance, is a complicated phenotype. It involves many symptoms, consisting of early morning smoking, heavier smoking, tolerance, and ease of relapse after quitting. Importantly, the development of ND is not a sudden event; it demonstrates a transition from experimental smoking with the first puff to regular smoking and finally to the establishment of ND (Bierut 2009). There are a series of assessment tools for ND; the more common are the Fagerström Test for Nicotine Dependence (FTND) (Heatherton et al. 1991) and the *Diagnostic and Statistical Manual for Mental Disorders* (4th edition; DSM-IV) (APA 1994). Although both scales are commonly used to evaluate the severity of ND, there is only a limited correlation between the two measures (Heatherton et al. 1991), because they focus on different aspects of ND. The FTND is a simplified measure compared with the DSM-IV, which lays particular emphasis on the number of cigarettes smoked per day (CPD) and the time between waking and the first cigarette, whereas DSM-IV emphasizes the behavioral and emotional aspects of addiction.

The first study concerning the contribution of variants in the *CHRNA5/A3/B4* cluster to ND was reported in 2007 (Saccone et al. 2007). Those authors examined 879 light smokers who had no symptoms of dependence, with an FTND score of 0, and 1050 heavy smokers, with an FTND score of >4.0, focusing on the transition from regular smoking to addiction. Among 3713 SNPs in more than 300 candidate genes analyzed, multiple risk SNPs were found in the *CHRNA5/A3/B4* gene cluster, with the most compelling evidence for a risk allele coming from a non-synonymous SNP, rs16969968, in *CHRNA5* ($P = 6.4 \times 10^{-4}$). Further, this SNP exhibited a recessive mode of inheritance, resulting in individuals with one copy of the risk allele A having a 1.1-fold increase in the risk of developing ND once exposed to cigarette smoking, whereas there was a twofold increase with the African-American (AA) genotype compared with subjects having no copy. Since then, numerous candidate gene-based analyses and large-scale GWAS have focused on the association of polymorphisms in the *CHRNA5/A3/B4* gene cluster with ND across different populations, leading to the conclusion that variants in this cluster do indeed contribute to the development of heavy smoking and ND (Amos et al. 2008; Bierut 2009; Bierut et al. 2008; Hung et al. 2008; Saccone et al. 2007; Stevens et al. 2008; Weiss et al. 2008).

Together, these studies demonstrate two groups of risk variants in the cluster. The first is marked by SNP rs16969968 in exon 5 of *CHRNA5*, which changes an aspartic acid residue into asparagine at position 398 (D398N) of the α5 subunit protein sequence or its tightly linked SNP rs1051730 in *CHRNA3*. The other is SNP rs578776 in the 3′-untranslated region (UTR) of *CHRNA3*, which has a low correlation with rs16969968 (see Table 5.1).

Further, the association of these SNPs with ND can be modified by different factors. For instance, Weiss et al. (2008) reported that individuals who became regular smokers before the age of 16 demonstrate a significant association between SNP

Table 5.1 Reported association of rs16969968, rs1051730, and rs578776 with nicotine dependence

dbSNP	Sample origin	Sample size	Cases (N)	Controls (N)	Minor allele frequency	Odds ratio	P value	References
rs16969968 (*CHRNA5*)	European (United States+Australia)	1929	1050	879	0.38	–	6.42E-04	Saccone et al. (2007)
	European (United States+Australia)	1929	1050	879	0.383	1.31	1.30E-04	Saccone et al. (2009a)
	Caucasian	1236	955	281	0.34	–	7.00E-03	Bierut et al. (2008)
	European American	1968	1093	875	0.378	1.37	6.30E-08	Stevens et al. (2008)
	European American	377	271	106	0.415	1.79	9.00E-04	Weiss et al. (2008)
	European American	2062	1063	999	0.35	1.4	4.14E-07	Saccone et al. (2009b)
	European (meta)	24,807	14,452	10,355	–	1.327	5.96E-31	Saccone et al. (2010)
	German (three cohorts)	5561	–	–	0.38	1.18	1.90E-04	Winterer et al. (2010)
	Mixed ethnic ancestry	571	–	–	0.357	–	<0.0001	(Sarginson et al. 2011)
	Caucasian	3441	–	–	0.41	–	1.10E-04	(Siedlinski et al. 2011)

(continued)

rs1051730 (*CHRNA3*)	United States+Australia	1929	1050	879	0.38	–	9.93E-04	Saccone et al. (2007)
	European (United States+Australia)	1929	1050	879	0.382	1.3	2.01E-04	Saccone et al. (2009a)
	Caucasian	1236	955	281	0.32	–	2.00E-02	Bierut et al. (2008)
	European American	1933	1073	860	0.378	1.37	9.30E-08	Stevens et al. (2008))
	European American	377	271	106	0.415	1.79	9.00E-04	Weiss et al. (2008)
	European American	2062	1063	999	0.349	1.4	5.88E-07	Saccone et al. (2009b)
	German (three cohorts)	5561	–	–	0.38	1.19	7.50E-05	Winterer et al. (2010)
	Mixed ethnic ancestry	571	–	–	0.358	–	<0.0001	Sarginson et al. (2011)
	Caucasian	3441	–	–	0.41	–	1.50E-04	Siedlinski et al. (2011)

Table 5.1 (continued)

dbSNP	Sample origin	Sample size	Cases (N)	Controls (N)	Minor allele frequency	Odds ratio	P value	References
rs578776 (*CHRNA3*)	United States+Australia	1929	1050	879	0.22	–	3.08E-04	Saccone et al. (2007)
	European (United States+Australia)	1929	1050	879	0.241	0.746	1.06E-04	Saccone et al. (2009a)
	Caucasian	1236	955	281	0.28	–	9.00E-03	Bierut et al. (2008)
	European American	1564	707	839	0.244	0.75	1.37E-06	Stevens et al. (2008)
	European American	377	271	106	0.218	0.6	4.80E-03	Weiss et al. (2008)
	European (meta)	22,915	13,391	9524	–	0.776	1.38E-25	Saccone et al. (2010)
	German (three cohorts)	5561	–	–	0.26	0.85	9.30E-04	Winterer et al. (2010)
	Mixed ethnic ancestry	571	–	–	0.331	–	2.40E-03	Sarginson et al. (2011)
	Caucasian	3441	–	–	0.24	–	2.00E-02	Siedlinski et al. (2011)

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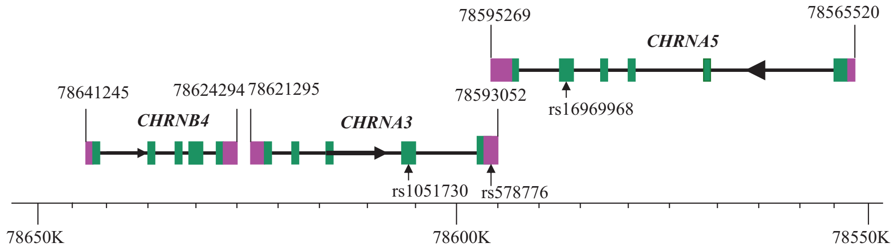

Fig. 5.1 Schematic diagram of the human *CHRNA5/A3/B4* cluster. *Horizontal black arrows* show the direction of transcription. *Green* and *pink rectangles* indicate exons and untranslated regions, respectively, while *horizontal black lines* represent introns. The positions of the genetic variants (rs1051730, rs578776, and rs16969968) significantly associated with ND are shown by vertical arrows

rs16969968 and the severity of nicotine addiction, whereas Grucza et al. (2010) found that the same SNP exhibited its effects mainly on late-onset smokers, after 16 years of age. What caused such inconsistent results remains to be investigated. In addition, other environment factors, such as parental monitoring (Chen et al. 2009), childhood adversity (Xie et al. 2012), and peer smoking (Johnson et al. 2010), influence the association between SNPs rs16969968 or rs1051730 and ND.

On the other hand, there are a few reports concerning the effect of common variants in *CHRNB4* on ND. Three independent GWAS meta-analyses revealed the importance of the *CHRNA5/A3/B4* gene cluster in influencing ND but failed to identify any SNP in the β4 receptor subunit gene as a contributor to the genetic association signal for heavy smoking (Liu et al. 2010; Thorgeirsson et al. 2010; Tobacco Genetics Consortium 2010). Thus, for the time being, we are not clear on whether common variants in *CHRNB4* play any role in the development of ND, although such a role is theoretically possible because of the high linkage disequilibrium (LD) patterns across *CHRNA5*, *CHRNA3*, and *CHRNB4* (Fig. 5.1).

3 Association Between Common Variants in the *CHRNA5/A3/B4* Gene Cluster and Smoking Initiation and Cessation

Cigarette smoking can be divided into three behaviors: initiation, ND, and cessation. Many variables influence the three processes, including age, education, social status, and so on. Although the variants in the *CHRNA5/A3/B4* gene cluster on chromosome 15 are strongly associated with ND and SQ, this region appears to play a smaller or less significant role in smoking initiation and cessation.

Thorgeirsson et al. (2008) reported that the variants in *CHRNA5/A3/B4* do not influence smoking experimentation and initiation. Similarly, Lips et al. (2010) and Kaur-Knudsen et al. (2011) concluded that the variants in the cluster on chromosome 15 do not play a role in identifying non-smokers and smokers. At the same

time, Maes et al. (2011) showed in a twin study that the SNPs associated with ND do not show a significant association with either smoking initiation or regular smoking. On the other hand, Sherva et al. (2008) reported an association between rs16969968 in the *CHRNA5* gene and enhanced pleasurable responses to initial cigarette smoking, suggesting that phenotypes related to subjective experiences during smoking experimentation may mediate the development of ND.

There are three main smoking cessation strategies: varenicline, nicotine replacement therapy (NRT), and bupropion (see Chap. 19 for further information). Each has its specific pharmacologic effects, and it is likely that one treatment will work for some people but not others with different genetic backgrounds. Studies of whether the variants in the *CHRNA5/A3/B4* cluster play a role in smoking cessation have reached inconsistent conclusions, with some studies demonstrating a significant role of SNPs in this gene cluster in quitting (Breetvelt et al. 2012; Breitling et al. 2009, 2010; De Ruyck et al. 2010; Lips et al. 2010), whereas others did not (Baker et al. 2009; Freathy et al. 2009; Munafo et al. 2011; Sarginson et al. 2011). Freathy et al. (2009) showed strong evidence of an association between rs1051730 and a greater likelihood of continued smoking during pregnancy, supporting a role of genetic factors in influencing smoking cessation at this important time. Furthermore, it was reported that variants in *CHRNA5* (rs16969968 or rs16969968-rs680244 haplotype) predict both ND and smoking cessation (Chen et al. 2012, 2014). They noted that the high-risk allele of rs16969968 is associated with a lower likelihood of quitting and, separately, a greater risk of cessation failure. However, in the placebo group or the group without any pharmacologic treatment, genetic variants do not predict abstinence across active treatment conditions. Thus, Chen and colleagues suggested that pharmacological cessation treatment might mitigate the genetic risks of cessation difficulty, which might be the explanation for the inconsistent results concerning smoking cessation and also should be considered in follow-up studies.

4 Association Between Common Variants in the *CHRNA5/A3/B4* Cluster and Lung Cancer

Lung cancer, which can be divided into two major histopathologic types (small-cell [SCLC] and non-small-cell [NSCLC] lung carcinoma), is the leading cause of cancer-related deaths throughout the world (Albuquerque et al. 2009). Among multiple risk factors for lung cancer, cigarette smoking is by far the most important, as many carcinogens are present in cigarette smoke; and others, such as NNK (4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone) and NNN (N′-nitrosonornicotine), are derived by metabolism from nicotine. These compounds can stimulate the growth or inhibit apoptosis of lung cancer cells.

In parallel with the studies of ND, several SNPs in the *CHRNA5/A3/B4* cluster seem to increase the risk of lung cancer, according to several GWAS and candidate gene-based association studies (Amos et al. 2010, 2008; Hung et al. 2008;

Thorgeirsson et al. 2008). Hung et al. (2008) first found that SNP rs16969968 was robustly associated with lung cancer after studying nearly 317,139 SNPs in 4614 subjects of European descent. Since then, this finding has been replicated in different ethnic populations (Amos et al. 2010; Jaworowska et al. 2011; Shiraishi et al. 2009; Timofeeva et al. 2011). However, whether the association of this SNP with lung cancer is directly or indirectly mediated by the variant's association with ND has been the subject of extensive debate in the past several years. One group favors a direct role of such variants, reasoning that the association was observed even in non-smokers (Hung et al. 2008) and remained significant after adjustment for SQ (Kaur-Knudsen et al. 2011; Wassenaar et al. 2011), whereas the other group, preferring an indirect role for the variant in lung cancer, argued that the studies failed to detect a significant association between the variant and lung cancer in never smokers (Girard et al. 2010). The inaccurate measurement of uptake of carcinogens using self-reported cigarettes per day (CPD) supports this view (Munafo et al. 2012).

There might have been some other elements, such as different ethnic backgrounds of the populations examined, sample sizes, and measurement strategies for smoking-related phenotypes, that contributed to the abovementioned conflict. For example, the populations used in most of these studies were of European origin (Amos et al. 2008; Hung et al. 2008), a group that has a 37–43% frequency of the rs16969968 A allele, whereas the A nucleotide is not detected or is uncommon in African, East Asian, and Native American populations (Bierut et al. 2008). Consequently, the association between variants in the *CHRNA5/A3/B4* gene cluster and lung cancer needs to be further investigated in well-designed studies.

5 Analysis of Rare Variants in the *CHRNA5/A3/B4* Gene Cluster

As mentioned above, multiple common variants in the *CHRNA5/A3/B4* cluster have been found consistently to be significantly associated with ND and smoking-related phenotypes. Among these, a non-synonymous change (rs16969968) in *CHRNA5* is the most strongly associated SNP in several GWAS (Bierut 2011; Thorgeirsson et al. 2010). Additionally, a group of highly correlated SNPs, specifically rs588765, increases *CHRNA5* mRNA expression, thus leading to a greater risk of ND (Saccone et al. 2010; Wang et al. 2009b). Despite these convincing results, only a small proportion of the variance (~5%) in smoking-related behaviors can be explained by these SNPs (Saccone et al. 2010). Rare variants, generally defined as those having a minor allele frequency of <1%, constitute another major part of genetic variants other than common ones. Thus, rare variants may well account for the inadequate explanation of the heritability of smoking-related traits, as identified by recent GWAS.

Although rare variants may play a critical role in developing or maintaining ND, the function of these variants in the *CHRNA5/A3/B4* gene cluster in the risk of ND has not been intensively investigated (Doyle et al. 2014). This is largely because

their low frequency in various populations increases the difficulties of ensuring adequate statistical power. Nevertheless, Wessel et al. (2010) recently studied the contribution of rare variants in nAChR subunit genes to FTND scores in treatment-seeking smokers and observed an association of rare SNPs in *CHRNA5* with the FTND score. This finding motivated Haller and her colleagues to study rare variants in other nAChR subunit genes in relation to ND (Haller et al. 2012). First, that research team undertook pooled sequencing of the coding and flanking sequences of *CHRNA5, CHRNA3*, *CHRNB4*, *CHRNA6*, and *CHRNB3* in AA and European American (EA) ND smokers and in light smokers without symptoms of dependence (Haller et al. 2012). Those investigators found that rare missense variants at conserved residues in *CHRNB4* (e.g., rs61737499 and rs12914008) or *CHRNA3* (rs8192475 in strong LD with rs12914008) are associated with a lower risk of ND and fewer CPD in both AAs ($P = 0.0025$ and $P = 6.6 \times 10^{-5}$, respectively) and EAs ($P = 0.023$ and $P = 0.021$, respectively) (Haller et al. 2012).

Using HEK293 cells, Haller et al. examined whether information from this type of functional testing of rare non-synonymous variants in *CHRNB4* can significantly improve the association between genotype and phenotype (Haller et al. 2014). Consistent with the results from Liang et al. (2005), the authors found that reduced sensitivity to activation by agonists (nicotine or ACh) results in a higher risk of ND and that, conversely, greater sensitivity reduces the risk. Moreover, an *in vivo* study has been conducted using animal models (Slimak et al. 2014) in which mice injected in the mHb with lentiviruses carrying the WT β4 subunit or β4 rare missense variants showed either aversion to or preference for nicotine, depending on the SNP. For instance, habenular expression of the β4 gain-of-function variant rs61737499 resulted in strong aversion, whereas transduction with the β4 loss-of-function variant rs56235003 failed to induce nicotine aversion. In sum, these functional studies demonstrate the vital role of rare variants in the *CHRNA5/A3/B4* gene cluster in smoking-related behaviors.

6 Functional Studies of the Compelling SNP rs16969968

To understand the molecular mechanism of the *CHRNA5/A3/B4* gene cluster associated with ND and lung cancer, one needs to determine which reported SNP might alter biological function. It appears that the most compelling SNP, rs16969968, is likely to be a biological contributor to ND, because it changes an amino acid in the α5 nicotinic receptor protein. This change is in the large cytoplasmic domain adjacent to the conserved amphipathic α-helix, so it is far from the extracellular acetylcholine binding site and unlikely to influence the sensitivity of agonist binding. In such a region, the negatively charged Asp398 might promote Ca^{2+} permeability, whereas Asn398, replaced by an amide group instead of the negatively charged carboxyl group, might inhibit it.

Consistent with this hypothesis, recent studies have demonstrated that the D398N polymorphism affects the function of $(\alpha4\beta2)_2\alpha5$ nAChRs (Bierut et al. 2008;

Kuryatov et al. 2011). When the two forms of the human α5 subunit (N398 and D398) are expressed in *Xenopus* oocytes, using α4 and β2 subunits as a concatemer structure, $(\alpha4\beta2)_2\alpha5$ nAChRs containing the allele of α5 associated with a greater risk of nicotine addiction exhibit diminished agonist-evoked intracellular calcium response, reduced calcium permeability, as well as enhanced short-term desensitization compared with $(\alpha4\beta2)_2\alpha5$ nAChRs possessing the major allele of α5 (Kuryatov et al. 2011). These results are qualitatively similar to those of an earlier study that involved expression in HEK293T cells of human α5 subunits with mouse α4 and β2 subunits (Bierut et al. 2008). The incorporation of α5 SNP into HEK293T cells transfected with α4β2 cDNA reduced the maximum response to a nicotinic agonist without altering its surface expression. However, these obviously different effects of rs16969968 are seen only on the $(\alpha4\beta2)_2\alpha5$ nAChRs; whether the SNP has a similar effect on the function of $(\alpha3\beta4)_2\alpha5$ nAChRs is unclear.

Morel et al. (2013) went a step further, adopting lentiviral re-expression vectors to achieve targeted expression of mutant α5 in the VTA of the brain using a knockin (KI) mouse model. Mice with the SNP rs16969968 in the VTA displayed intermediate behavioral and electrophysiological phenotypes compared with those of the α5 KO mice, suggesting that the non-synonymous α5 variant rs16969968, frequently present in subjects of European descent, exhibits a partial loss-of-function *in vivo*. This leads to higher nicotine consumption in the self-administration paradigm, thus defining a critical link between this SNP, its expression in VTA DA neurons, and nicotine intake.

There may be a second biologic mechanism in the *CHRNA5/A3/B4* gene cluster that is associated with heavy smoking and ND, including different extents of expression of *CHRNA5* mRNA in the brain (Wang et al. 2009a). Joint statistical analysis of the two loci (or called haplotype) demonstrates that the amino acid change through SNP rs16969968 and varying *CHRNA5* mRNA expression tagged by rs588765 (or rs578776 or rs3743078) independently contributes to ND. The risk allele of rs16969968 occurs primarily on the low mRNA expression allele of *CHRNA5*, whereas the non-risk allele of rs16969968 occurs on both high- and low-expression alleles tagged by rs588765 in *CHRNA5*. When the non-risk allele occurs against the background of low expression of *CHRNA5* mRNA, the risk of ND and lung cancer is significantly lower than in persons with higher mRNA expression (Fig. 5.2). Together, these studies reveal three levels of risk associated with *CHRNA5* and at least two distinct mechanisms conferring risk for ND: altered receptor function caused by rs16969968 and variability in *CHRNA5* mRNA expression.

However, there is another hypothesis, from a different perspective, to explain the vital function of SNP rs16966698. Hong et al. (2010) suspected that the smoking variance explained by the allele-modulated circuits was much higher than the smoking variance explained by the genotype alone, making brain circuit measures an intermediate marker for the convergent effects of genes. Thus, the α5 gene variant Asp398Asn is associated with a dorsal anterior cingulated ventral striatum/extended amygdale circuit, so that the Asn "risk allele" reduced the intrinsic resting functional connectivity strength in this circuit. At the same time, the findings from this work suggest a plausible circuit-level explanation for why rs16969968 and rs578776

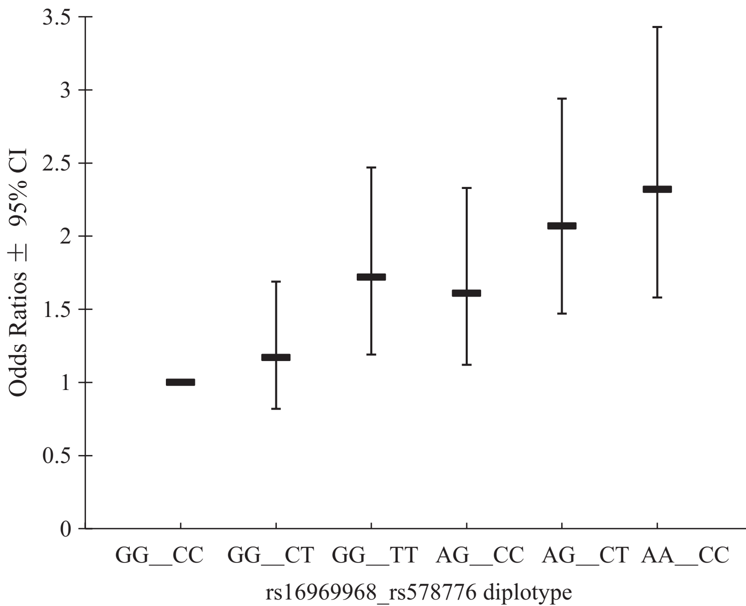

Fig. 5.2 Association of different rs16969968–rs588765 diplotypes with nicotine dependence. The bars represent odds ratios (±95% confidence intervals) using GG_CC as a reference. "A" indicates the risk allele of rs16969968, and "C" indicates the low mRNA expression allele of *CHRNA5* (Adapted from the report by Wang et al. 2009a, with the permission of Oxford University Press, license number 3416761480752)

represent two independent smoking-related signals in the *CHRNA5/A3/B4* cluster. The authors of this study distinguished the rs578776-related dACC-thalamus circuit, which appeared sensitive to the "state" of smoking, from the rs16969968-influenced dACC-ventral striatum circuit, predicting nicotine addiction severity.

7 From Association to Mechanism: Role of the α5 Subunit

To determine the function of the clustered nAChR subunits, knockout (KO) approach, especially KO rodent models, have been employed, primarily because of the lack of receptor agonists and antagonists with selectivity for all three subunits. So far, only α5 and β4 KO mice are available (Wang et al. 2002, 2003; Xu et al. 1999), and mice that do not express the α3 subunit usually die soon after birth as a result of multi-organ dysfunction (Xu et al. 1999). Thus, recent studies focus mainly on the function of the α5 and β4 subunits in determining the cause of the high risk of ND, with a special focus on the α5 subunit because of the functional SNP rs16969968.

The α5 nAChR subunit demonstrates a relatively discrete mRNA expression profile in the brain, with the highest densities of expression found in the mHb, which

projects almost exclusively to the IPN via the fasciculus retroflexus (De Biasi and Salas 2008; Sheffield et al. 2000). Recently, Fowler et al. (2011) adopted the α5 KO mouse model (analogous to individuals with reduced α5 receptor function) to examine the underlying mechanism of ND. These mice responded far more vigorously than wild-type (WT) mice to nicotine infusions at high doses and consumed significantly more nicotine than their WT littermates when tested under a progressive ratio schedule for reinforcement. Whereas the WT mice tried to control their nicotine intake through intravenous self-administration to achieve a consistent, desired blood concentration, KO mice did not, appearing to consume greater amounts of the drug as the dosage increased (Fig. 5.3). This finding leads to a hypothesis that deficient α5* nAChR signaling attenuates the negative effects of nicotine that limit its intake. Consistent with this result, the same manipulation in rats weakened the aversive effects of higher doses of nicotine but did not alter the reinforcing effects the drug on the brain reward system, as measured by nicotine-induced elevations and lowering of intracranial self-stimulation (ICSS) thresholds (Fowler et al. 2011). These findings are complemented by another study conducted by the same team (Fowler et al. 2013) employing a conditional place preference task to represent the differential effects of nicotine dose on reward in α5 KO and WT mice (Jackson et al. 2010). Fowler et al. (2011) demonstrated that the mHb-IPN pathway of the KO mouse is

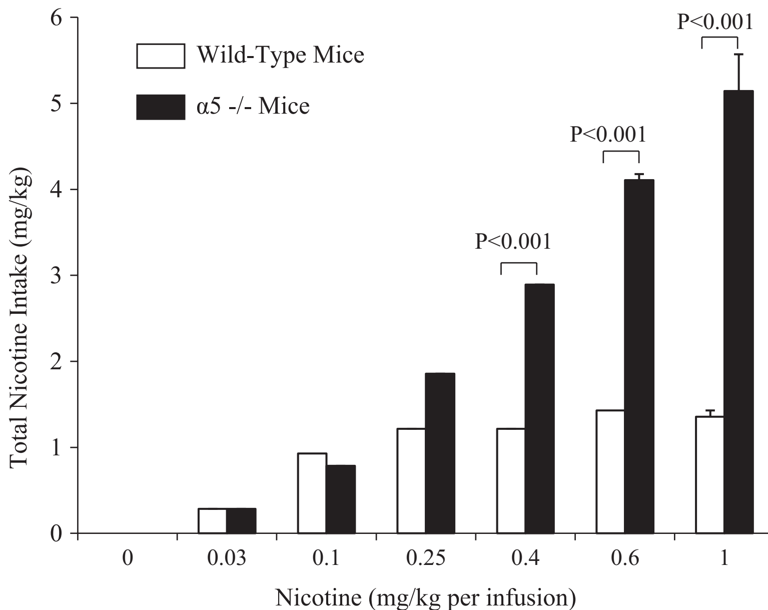

Fig. 5.3 Increased total nicotine intake (mg/kg) in $\alpha5^{-/-}$ mice compared with WT mice receiving infusions of high doses of nicotine. Data are presented as mean (± SEM) total nicotine intake at each dose. $P < 0.001$ indicates statistically significant differences between these groups at the same nicotine dose (Adapted from the paper by Fowler et al. 2011, with the permission of Nature Publishing Group, license number 3416820244064)

far less sensitive to nicotine-induced activation than that in WT mice by using Fos immunoreactivity as a measure of neuronal activation. RNA interference-mediated KO of the α5 nAChR subunit in the same rat brain region resulted in similar responses to nicotine (Fowler et al. 2011). Intriguingly, virus-mediated re-expression of the α5 nAChR subunit in the MHb-IPN pathway of the KO mice abolished the increased nicotine intake seen at higher doses (Fowler et al. 2011). Taken together, these findings indicate that the α5 receptor subunit is responsible for transmission of some aversive qualities of nicotine. In other words, nicotine-induced activation of the MHb-IPN pathway by the α5 receptor subunit results in a negative motivational signal that limits further nicotine intake. Hence, disrupted sensitivity of the MHb-IPN tract to nicotine in the α5 KO mouse diminishes this negative signal and induces greater nicotine intake.

In addition to the α5 nAChR subunit, evidence suggests that β4* nAChRs in the mHb-IPN pathway play a key role in regulating nicotine consumption. For example, Frahm et al. (2011) reported that mice overexpressing the β4 subunit as a result of bacterial artificial chromosome (BAC) transgenic technology consumed far less nicotine than their WT counterparts, and this effect could be reversed by lentiviral-mediated expression of the α5 D397N variant in the mHb (Frahm et al. 2011). These findings suggest that, similar to the α5 nAChR subunit, the β4 subunit regulates sensitivity to the aversive effects of nicotine, which controls the quantity of the drug consumed.

Apart from their role in the aversive effects of nicotine through the mHb-IPN pathway, the α5 and β4 nAChR subunits may have an action in nicotine withdrawal. Withdrawal symptoms can be divided into two classes: somatic and affective. The first are characterized in rodents by increased grooming, scratching, and shaking (Damaj et al. 2003; Kenny and Markou 2001), whereas the latter include primarily depressed mood, anxiety, difficulty concentrating, and so on in humans (Doherty et al. 1995; Kenny and Markou 2001; Parrott 1993). Withdrawal can be precipitated by administration of nicotine antagonists such as mecamylamine during chronic nicotine exposure. One study showed that chronically nicotine-treated β4 KO mice displayed significantly milder somatic withdrawal symptoms than WT mice when the symptoms were precipitated by mecamylamine (Salas et al. 2004). Furthermore, α5 KO mice that were dependent on nicotine (delivered through subcutaneously implanted osmotic minipumps) did not show somatic signs of nicotine withdrawal (Salas et al. 2009). Considering that β4* and α5* nAChRs are robustly expressed in the mHb-IPN pathway and that mecamylamine was infused directly into either the mHb or the IPN of nicotine-dependent WT mice, the precipitated expression of somatic withdrawal symptoms demonstrates that these two nAChR subunits and perhaps others enriched in the mHb-IPN pathway are critical for the expression of nicotine withdrawal. On the contrary, Fowler et al. (2013) concluded that the reward-inhibiting effects of precipitated nicotine withdrawal were not regulated by α5* nAChRs, given the fact that the magnitude to which mecamylamine-precipitated elevations of ICSS thresholds was similar in nicotine-dependent WT and KO mice (Fowler et al. 2013). Interestingly, another study (Jackson et al. 2008) showed that α5* nAChRs are more closely associated with physical signs of nicotine withdrawal than with affective symptoms, because chronic nicotine-treated α5 KO mice appeared anxious during withdrawal.

Addiction to cigarette smoking depends not only on attenuating the aversion to high doses of nicotine and nicotine withdrawal, as described above, but also on the reinforcing effects of low doses of nicotine: in other words, the balance between the reward and aversive action of the drug (Doherty et al. 1995; Kenny and Markou 2001). Furthermore, although the α5 nAChR subunit is most densely expressed in the mHb-IPN pathway, its expression also is detectable in many other addiction-relevant brain regions, for instance, a high percentage in the VTA, which underlies the rewarding and addictive properties of drugs of abuse through the dopaminergic (DAergic) neurons (Klink et al. 2001). Consequently, the α5* nAChRs are subjected to the same action in the VTA that explains their role in ND. However, many studies trying to identify the role of the α5 receptor subunit in the mHb-IPN pathway failed to find an effect in the VTA, especially in the DA neurons (Fowler et al. 2011, 2013). There is a first report that comprehensively analyzed the role of the α5 nAChR subunit in the VTA DA system (Morel et al. 2013). This study investigated the reinforcing effects of nicotine in drug-naive α5 KO mice by using an acute intravenous nicotine self-administration task and *ex vivo* and *in vivo* electrophysiological recording of nicotine-elicited DA cell activation. The fact that α5 KO mice, compared with WT mice, exhibited decreased sensitivity of the DAergic system and a dramatic shift to high nicotine doses in an acute nicotine injection paradigm (Morel et al. 2013) suggested a crucial role of α5* nAChRs in determining the minimum nicotine dose necessary for DA activation and thus nicotine reinforcement (Fig. 5.4). In addi-

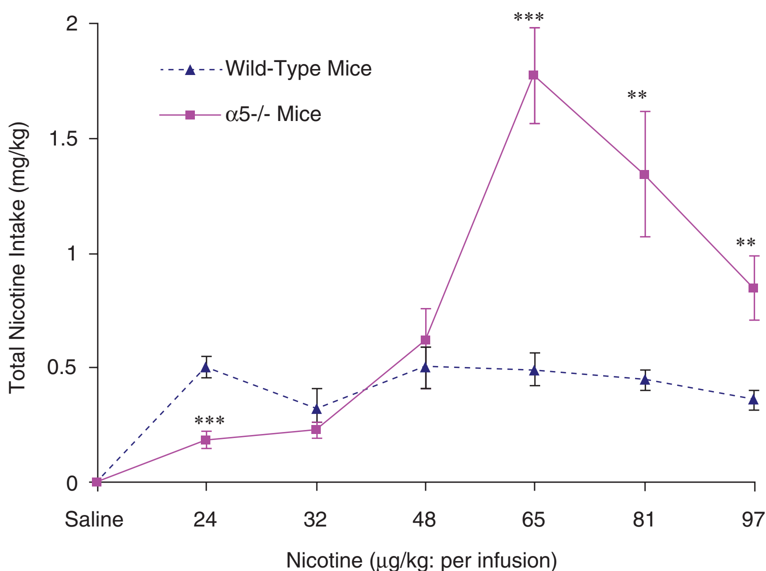

Fig. 5.4 Crucial role of α5* nAChRs in intravenous self-administration task (IVSA). $\alpha5^{-/-}$ mice exhibited a decreased sensitivity of the DA neurons and a dramatic shift to high nicotine doses compared with WT mice. Data are presented as mean (±SEM) total nicotine intake at each dose. ***$P < 0.001$, **$P < 0.01$ (Adapted from the publication by Morel et al. 2013, with the permission of Nature Publishing Group, license number 3416821041833)

tion, normal responses like those in WT mice were restored in KO mice by generalized lentiviral-mediated re-expression of the α5 subunit in all VTA cells or targeted to VTA DA cells specifically (Morel et al. 2013). These findings have defined novel, largely unexpected roles for the α5 nAChR subunit in reinforcing the effects of nicotine, although it acts only as an accessory subunit instead of contributing to the nicotine binding site. This aspect of the research may broaden our horizons in understanding the underlying mechanisms of the *CHRNA5/A3/B4* gene cluster in the development of ND, although independent verification of the findings is lacking.

8 Concluding Remarks

Research has implicated variants in the *CHRNA5/A3/B4* gene cluster on chromosome 15 in the development of ND. There is now a compelling body of evidence linking SNPs rs16969968 (or its strongly linked SNPs) and rs578776 (or rs588765) to smoking-related phenotypes. Joint statistical analyses of the two loci suggest the existence of two independent molecular mechanisms in ND. One is the amino acid change through SNP rs16969968, and the other is differing degrees of *CHRNA5* mRNA expression tagged by rs588765 (or rs578776, rs3743078). However, these findings reveal only a small portion of both common and rare variants in the *CHRNA5/A3/B4* cluster. Additional loci associated with smoking-related phenotypes await discovery. In particular, despite its difficulty, much attention should be paid to studies of rare variants in this gene region in order to understand in depth the genetics of ND.

There still is some controversy about the relation between the implicated SNPs and lung cancer, although the findings from GWAS are robust. Whether this association is direct or merely a by-product of ND must be investigated further. Because there have been no specific pharmacological reagents for the α5, α3, or β4 nAChR subunits that are useful in elucidating such complicated relations, design of highly specific nAChRs ligands is of prime importance. Alternatively, KI mouse model studies may directly examine the effects of variants given a constant carcinogen exposure. In other words, if, for example, SNP rs16969968 can be inserted into mice while ensuring that other conditions remain the same, the difference between the two groups of mice would be only in this SNP. Supposing that there is a difference in lung cancer rates between the two groups of mice, we can conclude that rs16969968 acts directly in the development of lung cancer. However, if not, we are more willing to believe that the SNP plays only an indirect role.

As with the rapid development of the large-scale GWAS, extensive genomic information concerning ND is now available. This lays emphasis on the urgency of understanding the biological mechanisms of how α5, α3, and β4 nAChR subunits modulate smoking-related behaviors, which presents both opportunities and challenges. Meanwhile, significant progress has been made in the past few years by using both in vitro and in vivo models, highlighting the importance of the α5 nAChR

subunit in regulating ND. However, these functional studies so far reveal only a critical role of the α5 subunit in controlling the aversive and withdrawal effects of nicotine. How the α3 or β4 nAChR subunits function in ND has not been clarified yet, primarily because of the smaller number of functional studies of these two subunits. Even though there are a few studies suggesting a role of the α5 subunit in the rewarding effect of nicotine, most of them remain to be validated in independent studies. Thus, more relevant studies are greatly needed in order to fully understand the underlying mechanisms of ND. Such a deep understanding of the mechanisms will improve the development of novel, tailored smoking cessation therapies.

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Chapter 6
Contribution of Variants in *CHRNB3/A6* Gene Cluster on Chromosome 8 to Smoking Dependence

Abstract Nicotine, the primary addictive compound in tobacco, plays a vital role in the initiation and maintenance of its use. Nicotine exerts its pharmacological roles through nAChRs, which are ligand-gated ion channels consisting of five membrane-spanning subunits. Besides the *CHRNA4*, *CHRNB2*, and *CHRNA5/A3/B4* cluster on chromosome 15, recent evidence from both GWAS and candidate gene-based association studies has revealed the crucial roles of the *CHRNB3/A6* gene cluster on chromosome 8 in ND. These studies demonstrate two distinct loci within this region. The first is tagged by rs13277254, upstream of the *CHRNB3* gene, and the other by rs4952, a coding SNP in exon 5 of that gene. Functional studies by genetic manipulation in mice have shown that α6*-nAChRs (where "*" indicates additional subunits), located in the ventral tegmental area (VTA), are of great importance in controlling nicotine self-administration. However, when the α6 subunit is selectively reexpressed in the VTA of the $\alpha6^{-/-}$ mouse by a lentiviral vector, the reinforcing property of nicotine is restored. To further determine the role of α6*-nAChRs in the process of nicotine-induced reward and withdrawal, genetic knockin (KI) strains have been examined, which showed that replacement of Leu with Ser in the 9′ residue in the M2 domain of α6 produces nicotine-hypersensitive mice (α6L9′S) with enhanced dopamine release. Moreover, nicotine-induced upregulation may contribute to the pathology of nicotine addiction, although the effect of chronic nicotine exposure on the expression of α6-containing receptors remains to be further investigated. This chapter presents the most recent studies concerning the genetic effects of the *CHRNB3/A6* gene cluster in ND.

Keywords Nicotinic acetylcholine receptors · *CHRNB3/A6* · Knockin · Knockout · Functional SNPs · Association · GWAS · Candidate gene · Minor allele frequency · Smoking addiction

1 Introduction

There are more than 4000 ingredients in cigarette smoke, but the pharmacological effects of smoking dependence are produced primarily by nicotine, which exerts its physiological roles through neuronal nicotinic acetylcholine receptors (nAChRs).

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The nAChRs, which are widely distributed in the central (CNS) and peripheral nervous systems, are ligand-gated ion channels consisting of five membrane-spanning subunits that can modulate the release of neurotransmitters and mediate fast signal transmission at synapses. Binding of nicotine to nAChRs forms the molecular basis for the reward obtained from nicotine and, eventually, the development of ND.

Different approaches, such as genetic, pharmacologic, and *in vitro* or *in vivo* functional studies, have been employed to link ND to one or more specific nAChR subunits (Rose 2007). Because of the wide distribution of α4β2* nicotinic receptors in the brain and their high affinity for nicotine, a large body of research has focused primarily on these subunits. Recently, several genetic variants located in nAChR subunit encoding genes other than *CHRNA4* or *CHRNB2* were detected by GWAS (Waters et al. 2003) and various candidate gene-based association and functional studies (Saccone et al. 2007; Bierut et al. 2008; Thorgeirsson et al. 2010). For example, the most compelling NB-linked SNP, rs16969968, in *CHRNA5* (Bierut 2011), leading to an amino acid change in position 398 (D398N) of the α5 subunit protein, has been consistently demonstrated to be a significant biological contributor to ND. For details on this part of the research progress, please refer to Chap. 5. It is believed that additional subunits or receptor subtypes are involved in the determination of different ND behaviors.

In this chapter, we discuss the evidence for a genetic association between variants in the *CHRNB3/A6* gene cluster on chromosome 8 and ND or ND-related phenotypes. Furthermore, some functional studies of α6 and β3 nAChR subunits using genetically engineered KO and KI mice are included.

2 GWAS of the *CHRNB3/A6* Gene Cluster and ND

In 2007, Bierut and her colleagues reported the first high-density association study on ND with the aim of identifying common genetic variants that contribute to the transition from occasional cigarette smoking to ND (Bierut et al. 2007). The sample consisted of 1050 heavy smokers, with a FTND score of >4.0, and 879 light smokers, who showed no symptoms of ND. Among 2.4 million SNPs examined, multiple risk SNPs were identified in the *CHRNB3/A6* gene cluster, with the most compelling evidence being seen for rs13277254 in *CHRNB3* ($P = 6.54 \times 10^{-5}$). In addition, another SNP, rs6474413, in complete linkage disequilibrium (LD) with rs13277254 in the same gene, was identified, with a P value of 9.36×10^{-5}. These nominal associations (based on the current genome-wide significance threshold of 10^{-8}) were subsequently replicated in a GWAS meta-analysis using the number of cigarettes smoked per day (CPD) as a measure of ND (Thorgeirsson et al. 2010).

Furthermore, Rice et al. (2012) reported that *CHRNB3* was more strongly associated with FTND than with CPD, indicating the importance of selecting an appropriate phenotype for association analysis. These authors carried out an independent GWAS with 1294 ND subjects (defined by FTND score) and 2071 non-ND controls

who had smoked at least one cigarette in their lifetimes, revealing that the genetic locus most strongly associated with ND was rs1451240 in *CHRNB3* (odds ratio [OR] 0.65; $P = 2.4 \times 10^{-8}$). Evidence for this association was strengthened in a subsequent joint meta-analysis with a previously published dataset (Saccone et al. 2007) (combined $P = 6.7 \times 10^{-16}$; total $N = 4200$). However, when CPD was used as the ND measure, the association no longer reached genome-wide significance, with a P value of 0.0007. These findings highlight the idea that phenotype selection is important in genetic association studies of ND.

3 Candidate Gene-Based Association Studies of the *CHRNB3/A6* Gene Cluster with ND

Besides the latest application of GWAS, significant efforts have been made to identify susceptibility loci for ND and its related phenotypes through a candidate gene approach with both case-control and family-based designs. After analyzing 3713 SNPs in more than 300 candidate genes for their association with ND, Saccone et al. (2007) reported that SNPs rs6474413 ($P = 9.36 \times 10^{-5}$) and rs10958726 ($P = 1.33 \times 10^{-4}$) in *CHRNB3* are significantly associated with ND. Both SNPs are located in the putative 5′ promoter region of the gene, with rs6474413 being 2 kb away from the start codon and 15 kb from rs10958726. Because of the high LD between the two SNPs, they may contribute to a single association signal. Using a sample of 1050 ND cases and 879 non-ND controls of European descent, the same population used in the study by Saccone et al. (2007), another study from the same group (Saccone et al. 2009) revealed a significant locus, tagged by rs13277254 at the 5′ end of *CHRNB3/A6* that is believed to influence the transition from smoking to ND. This finding was replicated in a follow-up study (Johnson et al. 2010), which considered peer smoking as a social environmental risk factor for smoking behavior. Because a set of common, highly correlated variants, which are tagged by rs6474413 and rs13277254 in the *CHRNB3/A6* gene cluster, have been associated with ND at the genome-wide significance level (Thorgeirsson et al. 2010; Bierut et al. 2007), significantly more effort has been focused on this region. Various SNPs in this cluster have a significant effect on ND and ND-related phenotypes in multiple ethnic populations (Tables 6.1 and 6.2).

On the basis of the previous association results of a high-density study covering the complete family of 16 *CHRN* genes in a population of European ancestry (Saccone et al. 2009), Saccone et al. extended their research to determine whether variants in the *CHRNB3/A6* gene cluster also are associated with ND in African-Americans (AAs) (Saccone et al. 2010). Those researchers did not detect any associated SNPs in their AA sample of 710 subjects. This suggests that there might be at least two distinct loci in the *CHRNB3/A6* gene cluster that are associated with ND in European-Americans (EAs). The first one was tagged by rs13277254, upstream of the gene cluster, together with additional associated SNPs in this region that con-

Table 6.1 Replicated SNPs in the *CHRNB3* gene cluster associated with ND-related behaviors

dbSNP ID	Sample origin	Sample size	Phenotype	Odds ratio or β value	Reported P value	Reference
rs4950	EA and Australian	1929	ND (FTND)	1.38	0.0001	Saccone et al. (2009)
	Ethnically diverse	1056	Subjective responses to tobacco (adverse, negative physical, positive)	4.88 8.13 12.25	0.02, 0.004, <0.001	Zeiger et al. (2008)
	Ethnically diverse	1524 families	Subjective responses to tobacco	NA	0.043	Zeiger et al. (2008)
	Caucasian, AA, and Hispanic	1051	Quit attempts	NA	0.021	Hoft et al. (2009)
	Caucasian, AA, and Hispanic	295	ND	4.62	0.007	Hoft et al. (2009)
	EA	2062	ND	0.78	0.00143	Saccone et al. (2010)
	EA, AA, and Asian (meta-analysis)	22,654	ND	0.1343	1.08E-05	Cui et al. (2013)
	Ashkenazi	591	Smoking status	1.94	9.8E-05	Bar-Shira et al. (2014)
rs10958726	EA and Australian	1929	ND (FTND)	NA	1.33E-04	Saccone et al. (2007)
	EA and Australian	1929	ND (FTND)	1.38	9.636E-05	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.126	0.005	Ehringer et al. (2010)
	EA	2062	ND	0.77	0.00113	Saccone et al. (2010)
	EA, AA, and Asian (meta-analysis)	22,654	ND	0.1546	1.24E-07	Cui et al. (2013)

(continued)

Table 6.1 (continued)

dbSNP ID	Sample origin	Sample size	Phenotype	Odds ratio or β value	Reported P value	Reference
rs13280604	Ethnically diverse	1056	Subjective responses to tobacco (adverse, negative physical, positive)	5.00 12.61	0.03, 0.001, <0.001	Zeiger et al. (2008)
	Ethnically diverse	1524 families	Subjective responses to tobacco	NA	0.011	Zeiger et al. (2008)
	Caucasian, AA, and Hispanic	1051	Quit attempts	NA	0.024	Hoft et al. (2009)
	Caucasian, AA, and Hispanic	295	ND	4.67	0.006	Hoft et al. (2009)
	EA, AA, and Asian (meta-analysis)	22,654	ND	0.1362	7.77E-06	Cui et al. (2013)
	Korean	576	NDSS (drive)	NA	0.03	Won et al. (2014)
rs6474413	EA and Australian	1929	ND (FTND)	NA	9.36E-05	Saccone et al. (2007)
	EA and Australian	1929	ND (FTND)	1.39	6.260E-05	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.114	0.011	Ehringer et al. (2010)
	EA	2062	ND	0.77	9.26E-04	Saccone et al. (2010)
rs13277254	EA and Australian	1929	ND (FTND)	1.4	4.022E-05	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.122	0.007	Ehringer et al. (2010)
	EA	2038	ND (FTND)	0.79	0.004	Johnson et al. (2010)
	EA	2062	ND	0.76	6.25E-04	Saccone et al. (2010)

(continued)

Table 6.1 (continued)

dbSNP ID	Sample origin	Sample size	Phenotype	Odds ratio or β value	Reported P value	Reference
rs6474412	EA and Australian	1929	ND (FTND)	1.38	1.126E-04	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.111	0.014	Ehringer et al. (2010)
	EA	2062	ND	0.78	0.00137	Saccone et al. (2010)
	EA, AA, and Asian (meta-analysis)	22,654	ND	0.1548	5.34E-07	Cui et al. (2013)
rs4952	EA and Australian	1929	ND (FTND)	NA	0.0163	Saccone et al. (2007)
	EA and AA	2772	ND	NA	0.00881	Saccone et al. (2010)
	EA and AA (meta-analysis)	5092	ND (FTND)	0.72	0.02	Culverhouse et al. (2014)
rs1955186	EA and Australian	1929	ND (FTND)	1.38	8.252E-05	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.119	0.009	Ehringer et al. (2010)
	EA	2062	ND	0.77	7.38E-04	Saccone et al. (2010)
rs1955185	EA and Australian	1929	ND (FTND)	1.38	1.010E-04	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.118	0.009	Ehringer et al. (2010)
	EA	2062	ND	0.78	0.00117	Saccone et al. (2010)
rs13277524	EA and Australian	1929	ND (FTND)	1.39	6.043E-05	Saccone et al. (2009)
	EA	1600	Early subjective response to tobacco (dizziness)	−0.121	0.007	Ehringer et al. (2010)
	EA	2062	ND	0.77	7.78E-04	Saccone et al. (2010)

(continued)

Table 6.1 (continued)

dbSNP ID	Sample origin	Sample size	Phenotype	Odds ratio or β value	Reported P value	Reference
rs4953	EA and Australian	1929	ND (FTND)	NA	0.0162	Saccone et al. (2007)
	Ethnically diverse	1056	Subjective responses to tobacco (adverse)	4.16	0.04	Zeiger et al. (2008)
rs4954	Han Chinese	48	ND (FTND)	2.18	4.25E-07	Wei et al. (2012)
	Korean	576	NDSS (drive)	NA	0.02	Won et al. (2014)

AA African-American, *CPD* cigarettes smoked per day, *EA* European-American, *FTND* Fagerström Test for Nicotine Dependence, *NA* not available, *ND* nicotine dependence, *NDSS* Nicotine Dependence Syndrome Scale

stitute Signal 1. Signal 2 is tagged by rs4952, the only known coding SNP in exon 5 of *CHRNB3*, which has a low correlation with rs13277254 (Fig. 6.1).

There are many other common variants in the *CHRNB3/A6* gene cluster that show a significant association with ND in multiple ethnic populations, including Han Chinese (Wei et al. 2012), AAs (Culverhouse et al. 2014), EAs (Stevens et al. 2008; Wang et al. 2014b), and Israelis (Greenbaum et al. 2006). We performed a meta-analysis of variants in *CHRNB3* in relation to ND by combining data from studies of subjects of different ethnicities (Cui et al. 2013). Although allele frequencies in the AAs were different from those in subjects of European and Asian ancestry, where the last two ethnic samples appeared similar, we found that the genetic effects of seven SNPs in *CHRNB3* are in the same direction among the three ethnic populations. More importantly, all these SNPs showed a significant association with ND. However, because of the different genetic structures of various ancestries, inconsistent results were found at the SNP level. We detected only four of seven SNPs in the samples of African origin, whereas the associations of all SNPs in the samples of European and Asian ancestry were significant (Cui et al. 2013). In contrast, none of these SNPs was reported to be associated with ND in studies in Finnish (Keskitalo-Vuokko et al. 2011), Swiss (Etter et al. 2009), or Czech (Hubacek et al. 2014) populations.

4 Association Studies of the *CHRNB3/A6* Gene Cluster with ND-Related Phenotypes

The early subjective response to tobacco smoking is a subphenotype of smoking initiation, which can predict later persistence of smoking as well as addiction. DiFranza et al. (2004) reported that greater sensitivity to nicotine during early

Table 6.2 Replicated SNPs in the *CHRNA6* gene cluster associated with ND-related behaviors

dbSNP ID	Sample origin	Sample size	Smoking measure	Odds ratio or β value	Reported P value	Reference
rs2304297	EA and Australian	1929	FTND	NA	0.00691	Saccone et al. (2007)
	Ethnically diverse	1056	Subjective responses to tobacco (positive)	0.170	0.003	Zeiger et al. (2008)
	Caucasian, AA, and Hispanic	1051	Quit attempts	NA	0.0044	Hoft et al. (2009)
	Mixed ethnic samples	6178	Response to tobacco taxation policy	−0.032	0.018	Fletcher (2012)
	Canadian	356	Dizziness at first inhalation of cigarette smoke	0.59	0.0057	Pedneault et al. (2014)
rs7828365	American	2847	CPD	0.84	0.036	Stevens et al. (2008)
	Canadian	356	Dizziness at first inhalation of cigarette smoke	0.58	0.0293	Pedneault et al. (2014)
rs9298628	Korean	576	NDSS (drive)	NA	0.02	Won et al. (2014)
	EA	2428	FTND	NA	2.18E-04	Wang et al. (2014a)
	EA and AA (meta-analysis)	7186	FTND	NA	0.00498	Wang et al. (2014a)
rs892413	Ethnically diverse	935	Smoking trajectories	−1.12	<0.001	Lee et al. (2013)
	EA	1730	CPD	NA	0.00769	Wang et al. (2014a)
	EA	2428	FTND	NA	5.30E-04	Wang et al. (2014a)
	EA and AA (meta-analysis)	7186	FTND	NA	0.00311	Wang et al. (2014a)

AA African-American, *CPD* cigarettes smoked per day, *EA* European-American, *FTND* Fagerström Test For Nicotine Dependence, *NA* Not Available, *ND* Nicotine Dependence, *NDSS* Nicotine Dependence Syndrome Scale

smoking attempts, as manifested by relaxation, dizziness, or nausea, was a determinant of later ND. Pomerleau et al. (2005) found that smokers who felt a pleasurable buzz during early smoking began to smoke much later than those who did not. Thus, it is reasonable to assume that genes, especially *CHRN*, associated with ND might play a role in early subjective responses to tobacco.

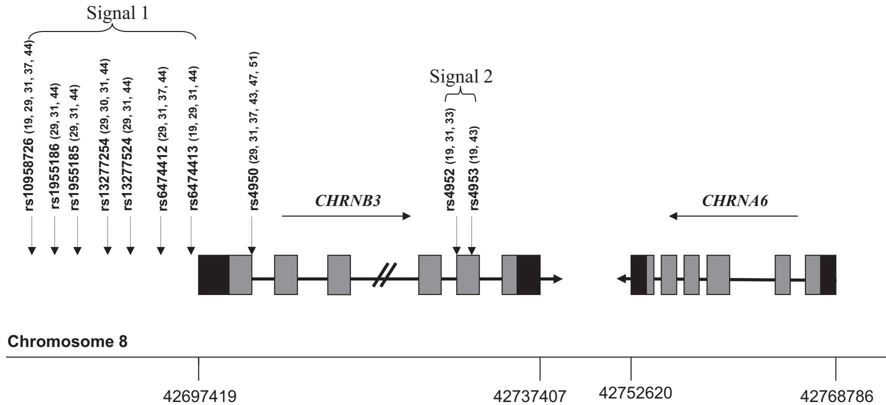

Fig. 6.1 Schematic diagram of the human *CHRNB3/A6* gene cluster. Horizontal black arrows indicate the direction of transcription. Gray and black rectangles mark exons and untranslated regions, respectively, while horizontal black lines represent introns (not drawn to scale). The genetic variants significantly associated with ND in EAs are shown by vertical arrows, which mark two distinct signals

The first report concerning the association between the variants in *CHRNB3/A6* and subjective responses to tobacco was published by Zeiger et al. (2008) using as subjects 1056 ethnically diverse adolescents and a separate community sample of 1524 families. The most significant associations were found between two *CHRNB3* SNPs (i.e., rs4950 and rs13280604) and three subjective response factors to initial tobacco use (adverse, negative physical, and positive). Since then, three studies (Ehringer et al. 2010; Hoft et al. 2011; Pedneault et al. 2014) have examined the association between variants in the *CHRNB3/A6* gene cluster and dizziness at first inhalation of cigarette smoke. Although both Ehringer et al. (2010) and Pedneault et al. (2014) have detected associations with several SNPs in the putative promoter region of *CHRNB3* and *CHRNA6*, Hoft et al. (2011) did not, which might be attributable to the small sample and the discrepancy of the phenotypic assessment tools used in these studies.

Apart from early subjective responses to tobacco, there exist many other ND-related phenotypes where the *CHRNB3/A6* gene cluster may play an important role, such as smoking status (never smoking vs. ever smoking) (Bar-Shira et al. 2014), smoking trajectories from early adolescence to adulthood (Lee et al. 2013), and various ND endophenotypes such as “novelty seeking” (Landgren et al. 2011) or “drive” (Won et al. 2014).

Additionally, smoking cessation is of great interest, because it is the ultimate goal of studying tobacco addiction and any other smoking-related phenotypes. Hoft et al. (2009) examined the association of SNPs in the *CHRNB3/A6* gene cluster with quit attempts in a nationally representative sample of households, which revealed that three SNPs upstream of *CHRNB3* (i.e., rs7004381, rs4950, rs13280604) and an SNP in the 3′ region of *CHRNA6* (rs2304297) were significantly associated with the number of unsuccessful quit attempts in Caucasian smokers. Further,

Fletcher (2012) provided novel evidence of the importance of genetics in explaining different responses to tobacco taxation policy. Individuals with the protective G/G polymorphism of rs2304297 in *CHRNA6* smoked less when there was high tobacco taxation, a response that may help with abstention, whereas others showed no response. The inability of this tobacco control policy (high taxation) to reduce the use of cigarettes in individuals with the C/C genotype suggests that alternative methods might be needed to improve smoking cessation in this population.

5 Association Analysis of Rare Variants in the *CHRNB3/A6* Gene Cluster

Both GWAS and candidate gene-based association studies have identified multiple common variants in the *CHRNB3/A6* gene cluster that contribute to ND and ND-related phenotypes. However, the role of rare variants (defined as those having a minor allele frequency [MAF] of <1%) of this cluster in ND has not been well investigated, largely because the extremely low MAF creates great difficulties in ensuring adequate statistical power. The only study of this topic was carried out by Haller et al. (2012), in which a DNA-pooling approach was used to sequence the coding and flanking regions of *CHRNA6* and *CHRNB3* in AA and EA ND smokers or smokers without any ND symptoms. In contrast to another study performed by the same group (Haller et al. 2014a), which showed that rare missense variants in *CHRNB3* were associated with a risk of alcohol and cocaine dependence, there is no evidence supporting the role of these variants in ND (Haller et al. 2012, 2014a).

Despite the absence of genetic association data for most SNPs, functional studies conducted by us indicated that rare variants in the hα6 subunit gene play a vital role in the etiology of ND (Dash and Li 2014). Although missense variations such as Asp57Asn (rs149966755) and Ser156Arg (rs373147726) and Asn171Lys (rs79945499) compromise the function of hα6*-nAChRs heterologously expressed in *Xenopus* oocytes, the nicotine sensitivity of these receptors is marginally or significantly increased by introducing Arg96His (rs188620180), Ala184Asp (rs200745568), Asp199Tyr (rs372469952), or Ser233Cys (rs369966241) variations into the hα6 subunit gene. Greater sensitivity to activation by agonists (nicotine or ACh) may result in a lower risk of ND, whereas reduced sensitivity increases the risk (Haller et al. 2014b). Individuals displaying altered α6*-nAChR pharmacology as a result of rare variants in *CHRNA6* are expected to exhibit different responses to cigarette smoking.

Because rare variants together with copy number variants (CNV) and small insertion/deletion polymorphisms (indels) constitute the majority of human genetic variations, they might contribute, at least partly, to the missing heritability of ND. Thus, we need to take rare variants into consideration when studying ND-related phenotypes, especially rare missense functional variants.

6 Functional Studies of the β3 and α6 Subunits by Genetic Manipulation in Rodents

As described above, numerous genetic studies have revealed a highly significant association between variants in the *CHRNB3/A6* gene cluster and greater vulnerability to ND (Thorgeirsson et al. 2010; Bierut et al. 2007; Rice et al. 2012), which generates a need to explore the underlying molecular mechanisms. However, to date, few pharmacologic ligands have been developed that target particular nAChR subtypes selectively. Therefore, to understand the contribution of α6 and β3 subunits to ND susceptibility in vivo and to circumvent the problem mentioned above, together with the difficulty associated with α6*-nAChRs in vitro expression, genetic manipulation in mice becomes invaluable. These manipulations generally include preventing the expression of the α6 or β3 subunit (KO) and replacing it with hyperactive derivatives (KI).

More attention has been paid to α6*- and β3*-nAChRs since the demonstration that these subunits exhibit an expression pattern restricted mainly to catecholaminergic and visual system neurons (Deneris et al. 1989; Forsayeth and Kobrin 1997; Vailati et al. 2000; Le Novere et al. 1996). By using transgenic mice expressing the α6 subunit fused with green fluorescent protein, the α6 subunit was found to be highly and selectively expressed in the VTA and substantia nigra pars compacta (SNc), regions important for the reinforcement of nicotine use (Mackey et al. 2012; Powers et al. 2013), with functional expression also in the locus coeruleus and retinal ganglion cells (Azam et al. 2002; Azam and McIntosh 2006). Immunoprecipitation and high-affinity [^{125}I]α-conotoxin MII (α-CtxMII)-binding studies showed that α6β2β3* and α6α4β2β3* pentamers are the predominant α6*-nAChRs in the striatum (Champtiaux et al. 2003; Zoli et al. 2002). Furthermore, the gene encoding the β3 subunit, which is adjacent to *CHRNA6* (Fig. 6.1), usually is co-expressed with α6. Because of the accessory role of the β3 subunit, it cannot form an acetylcholine-binding site, although it has an essential role in α6*-nAChR biogenesis and function (Cui et al. 2003; Gotti et al. 2005). Gotti et al. (2005) discovered that β3-subunit deletion dramatically reduced, but did not eliminate, α6*-nAChRs expression in the DA cell body (VTA) and terminal region (striatum), suggesting the importance of β3 for the correct assembly, stability, and transport of α6-containing receptors in dopaminergic neurons. In addition, a study conducted by Cui et al. (2003) demonstrated that disruption of the β3 gene does not affect expression of the mRNA for α6 or other subunits in the same brain areas. Those investigators also found that β3-KO mice have altered locomotor activity and prepulse inhibition (PPI) of acoustic startle responses, behaviors that are regulated in part by nigrostriatal and mesolimbic dopaminergic neurotransmission. Knowledge of these alterations is supported by the evidence that a population of β3-dependent nAChRs, which are sensitive to inhibition by α-CtxMII, modulates striatal dopamine release (Cui et al. 2003). In addition, Kamens et al. (2015) showed that the protective variant rs6474413 identified in human studies reduces expression of the *CHRNB3* subunit and decreases β3 gene expression during reduced nicotine intake in mice.

The α6-null mice grow normally and show no obvious developmental, neurologic, or behavior deficits (Champtiaux et al. 2002, 2003). By using autoradiography, Champtiaux et al. (2002) found complete disappearance of [^{125}I]α-CtxMII binding in both midbrain dopaminergic neurons and the visual system after deleting the α6 subunit, indicating that α6 is an essential component of the native binding site of this toxin. Another study (Pons et al. 2008) has shown the central role of α6 in the VTA in acute nicotine reinforcement.

The nicotine self-administration examination usually is conducted in 30 min with matched animal pairs placed in the experimental boxes, with one animal defined as active and the other as passive. Each nose poke (NP) by the active mouse activates the computer-operated syringe pump that delivers either nicotine or saline to both the active and the passive animals, whereas NPs by the passive mouse are recorded but have no scheduled consequences. By calculating the ratio between the number of responses of the active and passive mice, the reinforcing effects of nicotine can be determined. When tested in this way, α6 wild-type (WT) mice self-administered nicotine in a unit dose of 26.3 μg/kg/infusion (inf), whereas their α6-KO drug-naive littermates did not. The α6-KO animals did not self-administer nicotine even in an extensive range of lower (8.7–17.5 μg/kg/inf) and higher (35–52.6 μg/kg/inf) doses. Importantly, when the α6 subunit was selectively reexpressed in the VTA of α6−/− mice using a lentiviral vector, the reinforcing property of nicotine was restored (Fig. 6.2) (Pons et al. 2008). In intracranial SA experiments where learning is required, α6-KO mice showed a trend (although it was not significant) toward reduced nicotine self-administration compared with WT control mice (Exley et al. 2011). These findings demonstrate that the α6 subunit in the VTA is necessary to maintaining nicotine self-administration. By employing the latter model, Sanjakdar et al. (2015) showed that nicotine displayed a typical inverted U-shape conditioned place preference (CPP) response curve in the WT mice. Although the dose of 0.5 mg/kg nicotine led to a significant CPP in the WT mice, it failed to produce a CPP response in α6-KO mice. In contrast, the higher nicotine dose of 1.0 mg/kg resulted in preference scores in α6-KO mice that were significantly higher than those in α6 WT littermates (Fig. 6.3). The α6-KO mice exhibit a rightward shift in the nicotine dose–response curve compared with WT mice, indicating that the rewarding effect of nicotine is mediated by α6*-nAChRs. Pharmacologic blockade of the α6 subunit by selective antagonists (e.g., α-conotoxin MII) attenuates nicotine-induced CPP (Sanjakdar et al. 2015; Jackson et al. 2009), further supporting the vital role of α6 in nicotine reinforcement.

Although the KO mouse model is an essential research tool for understanding the mechanisms of ND, it typically allows addressing only questions of necessity, not sufficiency. To fully understand the diverse roles of different subunits or subtypes in the process of nicotine-induced reward and withdrawal, KI strains have been developed. Replacement of Leu with Ser in the 9′ residue in the M2 domain of the α6 subunit produces nicotine-hypersensitive mice. These α6L9′S strains show hyperactive locomotion and fail to habituate to a home cage, a novel environment, or reduced wheel rotations (Drenan et al. 2008, 2010; Grady et al. 2010; Cohen et al.

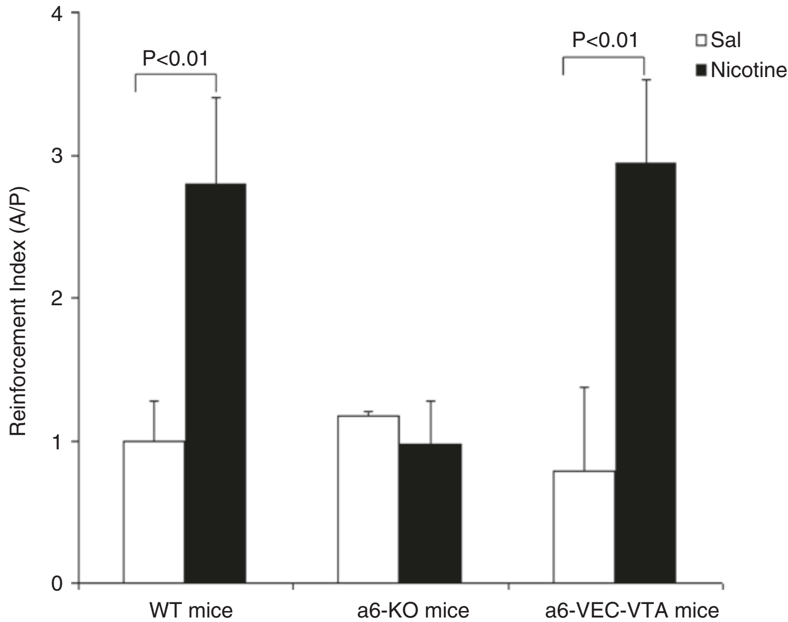

Fig. 6.2 Nicotine intravenous self-administration in WT and α6-VEC-VTA mice but not in α6-KO mice. Data are presented as mean (± SEM) reinforcement index (i.e., ratio of the cumulative nose pokes [NPs] by the active mice with respect to yoked control passive mice over the 30 min session in each group). The dose of nicotine was 26.3 μg/kg/inf. $P < 0.01$ indicates statistically significant differences between nicotine-treated and saline control groups (The data are adapted from Pons et al. 2008)

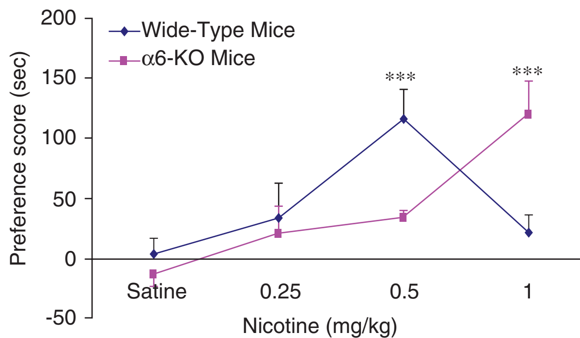

Fig. 6.3 Crucial role of α6* nAChRs in the rewarding effects of nicotine based on conditioned place preference. The α6-KO mice exhibited a rightward shift in the nicotine dose–response curve compared with WT littermates. Data are presented as mean (± SEM) preference score (sec). ***$P < 0.001$ (The data are adapted from Sanjakdar et al. 2015)

2012), which is consistent with enhanced dopamine neuron firing and release (Drenan et al. 2008, 2010; Cohen et al. 2012; Wang et al. 2014a). In addition, by crossing α4-KO mice with α6L9′S strains, it was found that the hyperactive effects caused by the gain of function mutation are mediated by α6α4* pentamers, because α6L9′S mice lacking the α4 subunit display essentially normal behavior (Drenan et al. 2010). Together, these studies demonstrate that α6L9′S mice are valuable in investigating the role of the α6 subunit in ND-related behaviors.

7 Effect of Chronic Nicotine Exposure on the Expression of α6-Containing nAChRs

Nicotine, like other substances of abuse, enhances dopamine transmission in the mesolimbic dopamine pathway, which is thought to play a critical role in the reinforcing effects that maintain smoking behaviors. Many studies on the rewarding effects of nicotine employed an acute administration approach. However, because smoking is a chronic behavior leading to long-term adaptive changes in the brain, knowledge of these chronic changes is essential for understanding ND and implementing measures that enable smoking cessation. Therefore, if genetic manipulation of nAChR genes in mouse KO or KI models represents a powerful research tool for identification of the particular contribution of specific receptor subunits to ND susceptibility, chronic nicotine treatment in vivo or in vitro, which mimics smoking in humans, is a valuable strategy.

After long-term nicotine exposure, high-affinity agonist binding to nAChRs in the CNS increases in both animal (Webster et al. 1999; Marks et al. 1992) and human (Perry et al. 1999) brains. This process, termed “nicotine-induced upregulation” (Hogg et al. 2003), may be involved in the pathology of nicotine addiction. An increase in [^{3}H]-Ach-binding sites was reported in the brains of smokers compared with non-smokers (Breese et al. 1997). The essence of nAChRs upregulation is related more to greater receptor numbers than to augmentation of the receptors’ affinity for nicotine (Buisson and Bertrand 2001). Furthermore, a hypothesis that nicotine acts as a pharmacologic chaperone to enhance a critical step inside the cell during the maturation of nAChRs has gained support (Lester et al. 2009). Specifically, nicotine binding to partially assembled nAChRs induces conformations that assemble more efficiently. This could be a compensatory response following desensitization of neuronal AChRs after chronic nicotine exposure (Picciotto et al. 2008; Fenster et al. 1999).

Accumulating studies have consistently observed upregulation by radiolabeled epibatidine, which identifies several nAChR subtypes in numerous brain regions after various nicotine treatments, including injection by osmotic minipumps or jugular cannula and infusion in drinking water (Marks et al. 1992; Rogers et al. 1998; Ryan et al. 2001; Sparks and Pauly 1999; Flores et al. 1997). Using [^{125}I]-epibatidine, A-85380, and cytosine, Nguyen et al. (2003) demonstrated that chronic exposure to

nicotine upregulates α4β2-containing receptors while having little effect on other nAChR subtypes. Nevertheless, α4β2*-nAChRs, which have wide distribution in the brain and a high affinity for nicotine, clearly become desensitized at an early stage of smoking behavior and thus do not function for most of the day in smokers. Despite the clarity of α4β2*-nAChR upregulation, it is not sufficient to explain continued smoking throughout the day (Rose 2007; Wooltorton et al. 2003). On the other hand, nAChRs with low affinity for nicotine (e.g., α7, α6) are not susceptible to rapid saturation and might play an important role in continued smoking. Besides α4β2-containing receptors, other diverse populations of nAChRs, such as α6β2* and α7*, have been identified in the mesolimbic dopamine pathway. These findings shed light on the vital importance of research on the upregulation of other nAChRs.

Unlike the situation with α4β2*-nAChRs, upregulation of α6-containing receptors in response to chronic nicotine exposure is controversial (Srinivasan et al. 2014). There have been reports of upregulation, downregulation, and no change from *in vitro* and *in vivo* experiments (Table 6.3). Upregulation of α6β2*- or α6β2β3*-nAChRs by incubation with nicotine was observed in cultured cell lines (Tumkosit et al. 2006; Walsh et al. 2008; Henderson et al. 2014), although functional expression of α6-containing receptors in a heterologous expression system proved to be difficult until some specific strategies were used, such as chimeras, gain of function mutagenesis, and so on. Unfortunately, in rodents, although Nguyen et al. (2003) and Parker et al. (2004) suggested upregulation of α6*-nAChRs in the nucleus accumbens, several other research groups (Lai et al. 2005; Perry et al. 2007; Doura et al. 2008; Perez et al. 2008; Marks et al. 2014) observed downregulation in the striatum. Interestingly, Perez et al. (2008) showed, by using the novel α-CtxMII analog E11A in α4-KO mice, that nicotine administration in drinking water for 2 weeks increased the α6(non-α4)β2*-nAChR population in the striatum, contrary to the reduction of total α6β2* subtypes in WT littermates. This leads us to hypothesize that α6α4β2* contributes to the downregulation in the striatum. Furthermore, in nonhuman primates such as the squirrel monkey, nicotine in the drinking water with a final concentration of 650 μg/ml for more than 6 months did not significantly change the α6β2*-nAChR binding site (McCallum et al. 2006; Perez et al. 2009, 2012) except in the study conducted by McCallum et al. (2005). This effect might be caused by region-specific actions, because earlier studies concentrated mainly on the nucleus accumbens, whereas the later ones focused on the striatum. Analyses in other reward-related regions of the brain also were performed, but this work has yielded no clear results or conclusions (Henderson et al. 2014; Parker et al. 2004; Mugnaini et al. 2006).

Several factors may account for these disparate findings. First, different treatment regimens with various concentrations of nicotine and exposure times were used. The importance of these changes is supported by evidence that α6β2β3*-nAChR shows upregulation after 50 nM nicotine treatment but downregulation with 500 nM nicotine (Henderson et al. 2014). Second, different species/cell lines, brain regions, and α6-containing subtypes may play a role in the inconsistent results. Last but not least, heterogeneity of the detection methods is an influencing factor, implying the urgency of developing more subunit-specific agonists and antibodies.

Table 6.3 Effect on the expression of α6- and β3-containing nAChRs by chronic nicotine exposure

Change	Species/ cells	Treatment/dose	Brain region	Subtype	References
Upregulation	Rat	Injection; 6.0 mg/kg/day; 2 weeks	NAcc; SC	α6β2*	Nguyen et al. (2003)
		Injection; 1.5 mg/kg/day; 18 days	NAcc; VTA/ SN; CPu; Thal	α6*	Parker et al. (2004)
	Mouse	Injection; 0.4 mg/kg/h; 10 days	VTA/SNc	α6*	Henderson et al. (2014)
		Injection; 2 mg/ kg/h; 10 days	VTA/SNc; mHb; SC	α6*	Henderson et al. (2014)
		Oral; 300 μg/ml; 2 weeks	Str	α6(nonα4)β2*	Perez et al. (2008)
	HEK tsA201 cell	Incubation; 100 μM; overnight	–	α6β2*; α6β2β3*;α6β4; α6β4β3*	Tumkosit et al. (2006)
		Incubation; 30 μM; 24 h	–	α6β2*	Walsh et al. (2008)
	Neuro-2a cell	Incubation; 50 μM; 24 h	–	α6β2β3*	Henderson et al. (2014)
No change	Monkey	Oral; 650 μg/ml; 6–8 months	NAcc	α6β2*	McCallum et al. (2006)
		Oral; 650 μg/ml; 8 months	VPu; DPu	α6β2*	Perez et al. (2009)
		Oral; 650 μg/ml; 3–6 months	NAcc	α6β2*	Perez et al. (2012)
	Rat	Injection; 6.0 mg/kg/day; 2 weeks	Str; SC	β3*	Perry et al. (2007)
			SC	α6*	
	Neuro-2a cell	Incubation; 50 μM; 24 h	–	α6β2*	Henderson et al. (2014)

(continued)

Table 6.3 (continued)

Change	Species/ cells	Treatment/dose	Brain region	Subtype	References
Downregulation	Rat	Oral; 650 µg/ml; 6 months	CPu; AcbC; AcbSh; SNPC; VTA	α6β2*	Mugnaini et al. (2006)
		Injection; 6.0 mg/kg/day; 2 weeks	Str	α6*	Perry et al. (2007)
		Injection; 6.0 mg/kg/day; 2 weeks	Str; DLG; VLG	α6*	Doura et al. (2008)
		Oral; 100 µg/ml; 2 weeks	Str	α6β2*	Perez et al. (2008)
		Oral; 25 µg/ml; 2–3 months	NAcc	α6β2*	Perez et al. (2013)
	Mouse	Oral; 300 µg/ml; 1–6 weeks	Str	α6*	Lai et al. (2005)
		Oral; 300 µg/ml; 2 weeks	Str	α6β2*	Perez et al. (2008)
		Injection; 0.125–4.0 mg/kg/h; 10 day	DLG; NAcc; Str; OT; VLG	α6β2*	Marks et al. (2014)
	Monkey	Oral; 650 µg/ml; 6 months	Str	α6*	McCallum et al. (2005)

AcbC core of nucleus accumbens, *AcbSh* shell of nucleus accumbens, *CPu* caudate putamen, *DLG* dorsolateral geniculate, *DPu* dorsal putamen, *HEK* human embryonic kidney, *NAcc* nucleus accumbens, *Neuro* neuroblastoma, *OT* olfactory tubercle, *SC* superior colliculus, *SN* substantia nigra, *SNPC* pars compacta of substantia, *Thal* thalamus, *VLG* ventrolateral geniculate, *VTA* ventral tegmental area mHb

8 Concluding Remarks

In this chapter, we have summarized several lines of evidence for the involvement of the *CHRNB3–CHRNA6* gene cluster in ND. A multitude of studies (GWAS and candidate gene-based association studies) analyzing various ND phenotypes have implicated variants in this gene cluster in the development of ND. The most compelling evidence is for SNPs rs13277254 and rs6474413 in *CHRNB3* as well as rs10958726 and rs1955186 within this same signal region. However, not much has been found specifically for the *CHRNA6* subunit gene, in contrast to its vital role in maintaining ND, as demonstrated with functional studies. These findings reveal only a small fraction of the variants, that is, these polymorphisms have small effects and can explain only a small proportion of the heritability of smoking-related behaviors. Therefore, additional loci (especially rare variants) need to be identified. Furthermore, despite the inconsistent results, it is important to study the genetics of ND in diverse populations. Differences in genetic architecture and allele

frequencies in different ethnic populations can help assign statistically significant signals to potentially causal variants.

Genetic modification of *CHRNB3* and *CHRNA6* in mice is a valuable approach to evaluating the contribution of each subunit to ND susceptibility. The KO mice display various behavioral phenotypes related to ND. For example, α6-KO mice do not self-administer nicotine, unlike their WT counterparts. In addition, studies in α6-hypersensitive (KI) mice are powerful in identifying compounds that activate or antagonize α6*-nAChRs as a means to improve the development of drugs for smoking cessation. Nevertheless, this approach is limited in the *in vivo* or *in vitro* studies focusing on elucidating the functional consequences of different SNPs. This investigation will provide significant insights into how genetic variations in humans underlie individual differences in the reinforcement, aversion, and withdrawal of nicotine. There exist significant differences in the pharmacologic properties of the α6 and β3 subunits, such as receptor upregulation after chronic nicotine treatment and differences among subtypes and brain regions. It remains to be determined how nicotine regulates the expression of α6*-nAChRs. Inconsistent results in different studies are likely a consequence of the unpredictable behavior of heterologous expression systems. Functional expression of WT α6*-nAChRs is difficult to achieve unless some modifications are adopted, for instance, subunit chimeras, concatameric subunits, and point mutagenesis of the α6 or β3 subunits. In spite of the significant progress, there still are many obstacles to be overcome. That may be why conflicting results concerning upregulation of α6-containing receptors occur in relatively few studies. Thus, advancing the heterologous expression of α6* receptors should be another focus of future research.

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Chapter 7 Genetic Contribution of Variants in GABAergic Signaling to Nicotine Dependence

Abstract Although genetics contributes significantly to tobacco smoking, the susceptibility genes and variants underlying this behavior remain largely unknown. Genome-wide linkage and association studies have implicated a number of genes and pathways in the etiology of ND. In this chapter, we focus on current evidence, primarily from human genetic studies, supporting the involvement of genes and variants in the γ-aminobutyric acid (GABA)ergic signaling system in the etiology of ND, based on the results from linkage, association, and gene-by-gene interaction analyses. Current efforts aim not only to replicate these findings in independent samples but also to identify which variant contributes to the detected associations and through what molecular mechanisms.

Keywords γ-Aminobutyric acid · GABA · Nicotine dependence · $GABA_A$ receptor · $GABA_B$ receptor · Linkage study · Association study · *GABBR2* · Pathway analysis · Genetic effect · Interaction

1 Introduction

Of the important neurotransmitters in the central nervous system (CNS) implicated in tobacco smoking, GABA is the main inhibitory one. Its modulatory actions are mediated through two types of receptors: the ionotropic $GABA_A$ receptor and the metabotropic $GABA_B$ receptor (Bettler et al. 2004; Vlachou and Markou 2010). The $GABA_A$ receptors form ion channels, whereas $GABA_B$ receptors activate second-messenger systems through G-protein binding. The GABA neurons are part of the mesolimbic dopamine system, critically important in mediating the reinforcing properties of drugs of abuse. Additionally, the GABA system is diffusely expressed in the brain; therefore, areas other than the mesolimbic system may be partly responsible for these effects. Considering the functional importance of GABAergic signaling in the CNS, the genes involved in the system have received great attention in human genetic study of addictions, including ND. The primary objective of this chapter is to provide an updated review of what we have learned from genetic epidemiologic studies on the involvement of genes in the GABAergic signaling system in drug addiction.

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2 Evidence for the Involvement of Genes in GABAergic Signaling in ND Based on Linkage Studies

During recent years, a significant number of genome-wide linkage studies have been reported on addiction to nicotine, alcohol, and other abused substances (Li and Burmeister 2009), especially for smoking-related behaviors, for which more than 20 such studies have been published (see Chap. 4). By examining the linkage regions reported in each study and applying the rigorous criteria proposed by Lander and Kruglyak (1995), 14 regions, located on chromosomes 3–7, 9–11, 17, 20, and 22, were found to show "suggestive" or "significant" linkage in at least two independent samples (Li 2008; Yang and Li 2016). Of them, the regions on chromosomes 9, 10, 11, and 17 have received the strongest support, with the regions on chromosomes 9 and 17 being the most interesting, given the primary objective of this chapter (Bergen et al. 1999; Bierut et al. 2004; Gelernter et al. 2007; Li et al. 2003, 2006).

3 Evidence for Association of GABA$_B$ Receptor Subunit 2 (*GABBR2*) with ND

On the basis of the linkage results showing a "suggestive" linkage on chromosome 9 with ND (Fig. 7.1a), reported initially by our group in the Framingham Heart Study (FHS) sample (Li et al. 2003) and verified in independent samples by us (Li et al. 2006) and others (Bergen et al. 1999; Bierut et al. 2004; Gelernter et al. 2004), we conducted positional candidate gene-based association studies on this region for several candidate genes in the Mid-South Tobacco Family (MSTF) sample (Beuten et al. 2005, 2007; Li et al. 2007, 2009). The first possibly relevant gene identified in this linkage region was the subunit 2 gene for the GABA$_B$ receptor (*GABBR2)* (Beuten et al. 2005). Since this report, we have genotyped more SNPs from *GABBR2* in large MSTF samples, in which we not only confirmed our earlier finding that *GABBR2* is significantly associated with ND but also showed that genetically determined vulnerability to ND is different in subjects of European and African origin (Li et al. 2009).

The GABA$_B$ receptor inhibits neuronal activity through G-protein-coupled second-messenger systems, which regulate the release of neurotransmitters and the activity of ion channels and adenylyl cyclase (Kaupmann et al. 1998; Vlachou and Markou 2010). Although they have not revealed the detailed mechanisms of the involvement of GABA$_B$ receptors in ND, preclinical studies have implicated GABAergic receptors in the rewarding effects of drugs of abuse, including nicotine (Corrigall et al. 2000).

To determine the genetic contribution of *GABBR2* variants to the detected linkage signal on chromosome 9, we performed two rounds of linkage analysis, with the first being considered a regular analysis without correcting for *GABBR2* SNPs and

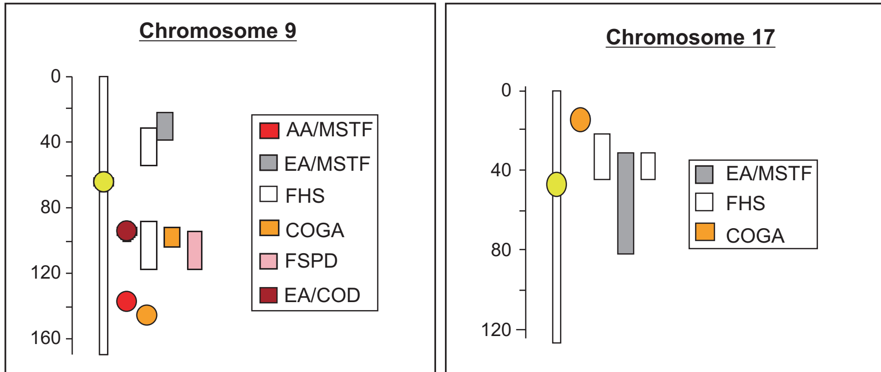

Fig. 7.1 Chromosomal locations of nominated regions on chromosomes 9 and 17 for all smoking-related measures with "significant" or "suggestive" linkage scores. The linkage results were obtained from the following studies: *AA/MSTF* African-American sample of the Mid-South Tobacco Family study (Li et al. 2006), *EA/MSTF* European-American sample of the Mid-South Tobacco Family study (Li et al. 2008), *FHS* Framingham Heart Study (Li et al. 2003; Wang et al. 2005), *EA/GCOD* European-American sample of Genetics of Cocaine or Opioid Dependence study (Gelernter et al. 2007), *COGA* Collaborative Studies on the Genetics of Alcoholism (Bergen et al. 1999; Bierut et al. 2004; Duggirala et al. 1999), *FSPD* Family Study of Panic Disorder (Gelernter et al. 2004)

the second a "justified" linkage analysis including *GABBR2* SNPs as covariates (Li 2006). As shown in Fig. 7.2, we found that the inclusion of *GABBR2* SNPs as a covariate reduced, but could not completely eliminate, the linkage signal detected on chromosome 9. The inclusion of *GABBR2* SNPs decreased the linkage signal on this chromosome by 36.5%, 27.7%, and 38.2% for smoking quantity (SQ), the Heaviness of Smoking Index (HSI), and the Fagerström Test for ND (FTND), respectively. These results indicate that *GABBR2* is indeed a candidate gene for a contribution to the ND linkage signal on chromosome 9 detected in our earlier study and that there must be other candidate genes in this region that contribute to the linkage signal we detected. This is because *GABBR2* SNPs explained only 27.7–38.3% of the linkage signal on chromosome 9. Indeed, our further positional candidate gene-based association analyses of this genomic region revealed that neurotropic tyrosine kinase receptor 2 (*NTRK2*) and Src homology 2 domain-containing transforming protein C3 (*SHC3*) are significantly associated with ND in the MSTF samples (Beuten et al. 2007; Li et al. 2007).

4 Interaction of *GABBR1* and *GABBR2* in Affecting ND

Like any other complex trait, nicotine addiction is controlled by multiple genetic factors, with each having a relatively modest effect, and by environmental factors, as well as by both gene–gene (epistatic) and gene–environment interactions (Flint

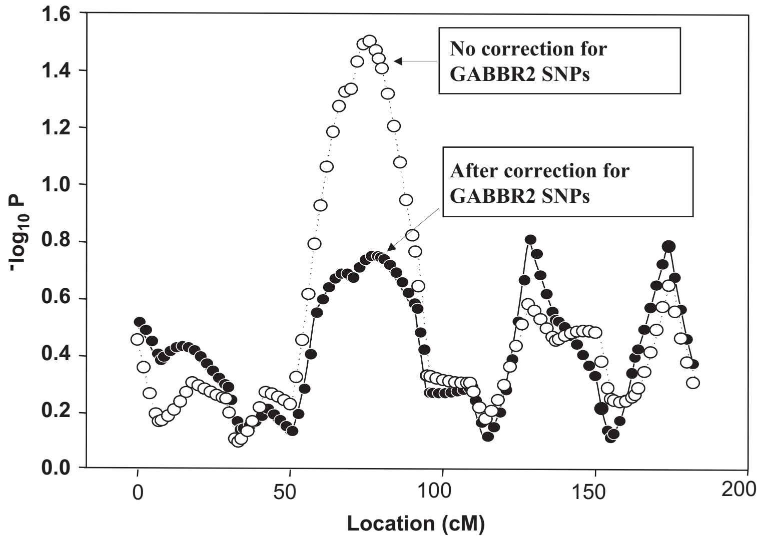

Fig. 7.2 Determination of contribution of *GABBR2* SNPs to linkage signal detected on chromosome 9

and Munafo 2008; Ho et al. 2010; van der Zwaluw and Engels 2009). As documented in other chapters in this book, significant efforts have been made to find vulnerability genes for ND. However, these approaches are effective only for genes with moderate to significant effects. The ability to identify susceptibility genes for ND has been improving but remains considerably limited by the presence of a diverse array of factors such as epistatic interaction, small–modest genetic effects, small samples, and heterogeneities. Detecting gene–gene and gene–environment interactions thus is more challenging (Flint and Munafo 2008; Ho et al. 2010; van der Zwaluw and Engels 2009).

In the search for determinants of gene–gene interaction, extensive efforts have been expended. Several combinatorial approaches, such as the multifactor dimensionality reduction (MDR) (Ritchie et al. 2001), the combinatorial partitioning method (CPM) (Nelson et al. 2001), and the restricted partition method (RPM) (Culverhouse et al. 2004), are promising tools for detecting gene–gene and gene–environment interactions. Since the original report, MDR has been most widely applied to detect interactions underlying a spectrum of complex disorders. However, these methods have critical limitations that restrict their practical use. For example, none of them allows adjustments for covariates. Also, MDR is applicable only to dichotomous phenotypes, and CPM and RPM cannot handle categorical phenotypes. To overcome the limitations of these established combinatorial approaches and to meet research needs in determining gene–gene and gene–environment interactions for complex phenotypes, a generalized MDR (GMDR) and a pedigree-based GMDR (PGMDR) have been developed for case-control (Lou et al. 2007a) and family-based (Lou et al. 2008) studies, respectively. These techniques permit adjustments

for discrete and quantitative covariates and are applicable to both dichotomous and continuous phenotypes. A detailed description of gene-by-gene interaction analysis is given in Chap. 11.

Specifically, regarding gene–gene interaction for the GABAergic signaling system for ND, using the PGMDR software, we detected significant interactive effects between the variants in *GABBR1* and *GABBR2* in ND (Li et al. 2009). This is noteworthy in that a relatively weak association of *GABBR1* with ND has been detected (Li et al. 2009) and indicates that a significant interaction exists between variants of *GABBR1* and *GABBR2* in affecting ND. Involvement of *GABBR1* in modulating ND risk is most likely through its interaction with *GABBR2*, where *GABBR2* polymorphisms directly alter the susceptibility to ND (Li et al. 2009). The reason for failing to detect a significant association of *GABBR1* itself with ND may be a strong dependence of *GABBR1* effects on specific *GABBR2* variants or a relatively small marginal effect of *GABBR1* variants in the samples studied. More importantly, a significant interaction of *GABBR1* with *GABBR2* in humans confirms previous findings of pharmacologic studies that showed that the $GABA_B$ receptor functions as a heterodimer of $GABA_{B1}$ and $GABA_{B2}$ subunits (Bettler et al. 2004; Vlachou and Markou 2010).

The involvement of the $GABA_B$ receptor in ND has been reported in many studies using animal models (Bettler et al. 2004), including a recently described genetic report on zebrafish in which a nicotine behavioral assay in a forward screening of genes altered by a gene-breaking transposon mutagenesis approach was taken (Petzold et al. 2009). This study used transposons in mutant zebrafish and screened for changes in the nicotine-induced locomotive response. It generated two mutant fish lines with significantly attenuated nicotine locomotive responses: *dbav* and *hbog*, which have mutations in the chaperonin-containing protein 8 (*cct8*) and a $GABA_B$ receptor ortholog, *gabbr1.2*, respectively. This identification of $GABA_B$ receptor involvement in the nicotine response of zebrafish provides further evidence for the role of the GABAergic system in the etiology of ND (Klee et al. 2010). In considering a consistent relation between reduced reward sensitivity and addiction, these findings point to a potential genetic basis for the involvement of $GABA_B$ receptor signaling in the etiology of ND.

5 Evidence for Association of Other Genes in the GABAergic System with ND

There is another candidate gene, called $GABA_A$ receptor-associated protein (*GABARAP*), that is located in a "suggestive" linkage region on chromosome 17 (see Fig. 7.1b) for ND or other smoking-related behavior (Duggirala et al. 1999; Li 2008; Li et al. 2003; Wang et al. 2005). *GABARAP* belongs to a family of microtubule-associated proteins that includes *GABARAP*, $GABA_A$-receptor-associated protein-like 1 (*GABARAPL1*), *GABARAPL2*, the yeast protein Apg8p/

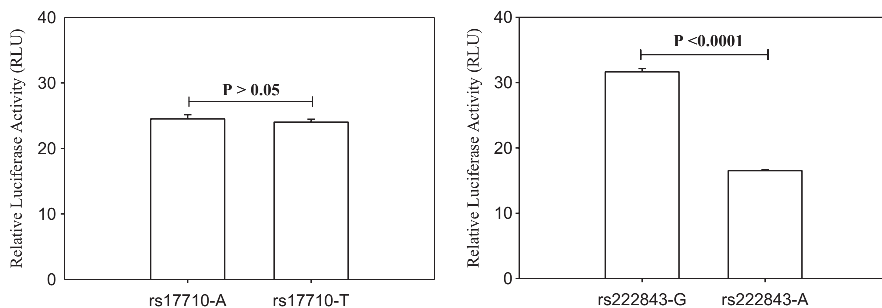

Fig. 7.3 Determination of allelic-specific expression of SNPs rs222843 and rs17710 in *GABARAP*. The SNPs rs222843 (G/A) and rs17710 (A/T) are located in the promoter and 3′-UTR of *GABARAP*, respectively. Using a luciferase reporter assay, we revealed a significant expression difference between the G and the A alleles of rs222843 ($P < 0.0001$) but not in the A and the T alleles of rs17710. Data are shown as mean ± SD ($N = 4$). **$P < 0.01$; paired student's *t*-test

Aut7, and light chain 3 of microtubule-associated protein 1 (*MAP1-LC3*) (Kabeya et al. 2000; Lang et al. 1998; Pellerin et al. 1993; Sagiv et al. 2000; Wang et al. 1999). Of the members of this family, *GABARAP* has been investigated extensively and found to interact with the γ2 subunit of the $GABA_A$ receptor. Such interactions among $GABA_A$ receptor, *GABARAP*, and tubulin promote clustering of the receptor, alter its channel kinetics, and enhance its trafficking to the plasma membrane in neurons (Chen et al. 2000; Leil et al. 2004; Wang et al. 1999). Furthermore, our microarray study indicated that *GABARAPL2* is highly regulated by nicotine in multiple rat brain regions in a time- and region-dependent manner (Li et al. 2004).

Through a two-stage fine-mapping approach on the basis of linkage analysis findings, we found that two SNPs (rs222843 and rs17710) in *GABARAP* are significantly associated with ND in European-American smokers (Lou et al. 2007b). Considering that SNPs rs222843 and rs17710 reside in the promoter and the 3′-untranslated region of *GABARAP*, respectively, we were interested to determine whether they are capable of regulating *GABARAP* expression. Using a luciferase reporter assay in human embryonic kidney HEK293 cells, we found that the promoter containing the G allele of rs222843 produced a nearly twofold increase in luciferase activity compared with the one containing the A allele (Fig. 7.3a). In contrast, we detected no difference in the expression of the chimeric reporters containing the A and T alleles of rs17710 (Fig. 7.3b). This indicates that rs222843, not rs17710, is functional in causing expression divergence of *GABARAP*. However, whether this differentially allelic-specific expression can be detected in human smokers remains to be examined further for this functional *GABARAP* variant.

Given the compelling evidence that *GABRA2* on chromosome 4 is significantly associated with alcoholism (Covault et al. 2004; Edenberg et al. 2004; Fehr et al. 2006; Lappalainen et al. 2005) and polysubstance dependence (Agrawal et al. 2006, 2008a; Drgon et al. 2006), Bierut et al. (2007) and Saccone et al. (2007) investigated whether genes in the $GABA_A$ gene cluster are associated with ND using a sample consisting of 1050 nicotine-dependent subjects (FTND ≥4) and 879 nondependent smoking controls (FTND = 0) who had smoked more than 100 cigarettes in their

lifetimes drawn from the Nicotine Single Nucleotide Polymorphisms (NICSNP) study. These studies revealed a significant association of SNPs in *GABRA4* (GABA receptor alpha 4), *GABRA2*, and *GABRE* (GABA receptor epsilon) with ND (Agrawal et al. 2008a). Furthermore, a linkage study has implicated the region near *GABRA2* on chromosome 4 in the etiology of cannabis use (Agrawal et al. 2008b).

6 Evidence for Involvement of GABA Receptor Signaling in ND Based on Pathway Analysis

As mentioned above, both linkage and association analyses have revealed several genes in the GABAergic signaling pathway that are associated with ND or other smoking-related behaviors. However, another study has failed to replicate some of those associations (Agrawal et al. 2008a). Many factors might contribute to difficulty in replicating the findings of linkage and association analyses, which include the presence of substantial heterogeneity, underpowered samples, small genetic effects, inconsistency in defining and assessing the phenotypes of interest, and different study designs and methods (Ho et al. 2010; Li 2008; Wang and Li 2010). Generally speaking, a conventional single-gene-based association study reports only the top-ranking SNPs or genes with the smallest statistic and has serious limitations because of functionally critical susceptibility SNPs/genes for a complex trait generally with subtler effects and overconservative multiple testing correction (Wang et al. 2007). To overcome these limitations, pathway-based association analysis has been proposed (Holmans et al. 2009; Wang et al. 2007), which examines the cumulative impact of a group of genes with modest individual contributions in the same pathway on a phenotype of interest. Compared with single-gene-based analysis, pathway-based analysis is supposed to reveal more convincing findings, and such findings should be more biologically plausible because a significantly enriched pathway presumably defines a more precise and more specific biological function than a single gene with multiple functions (Holmans et al. 2009). Further, given the fact that Bonferroni correction is considered to be overly conservative for multiple testing and that genes with subtler effects could hardly survive such a correction in large-scale association studies, the pathway-based analysis offers an attractive and potentially powerful alternative perspective – a “two-step” testing procedure that first identifies significant clusters of genes and then tests pathways within each significant group.

To identify pathways associated with ND and its related behaviors, we recently conducted a comprehensive pathway-based association analysis for three important smoking-related behaviors: smoking initiation, ND, and smoking cessation (Wang and Li 2010). By searching the literature on genetic studies for the behaviors, including both candidate gene-based and genome-wide association studies, we identified most, if not all, genes that have been reported to be associated with these phenotypes. We then applied various pathway-based approaches to these genes, which revealed 9, 21, and 13 enriched pathways among the genes associated with smoking initiation, ND, and smoking cessation, respectively. Of these pathways, we found that GABAergic

signaling is significantly associated with ND (Wang and Li 2010). Moreover, we found significant genetic overlap among these three smoking-related phenotypes.

7 Concluding Remarks

In sum, significant progress has been made in identifying susceptibility loci and genes for tobacco smoking. On the basis of the identified linkage peaks on chromosomes 9 and 17 and prior knowledge of the biological functions of the products of each gene, variants in *GABRA4*, *GABRA2*, *GABRE*, *GABBR2*, and *GABARAP* are significantly associated with ND. Linkage peaks on chromosomes 4 and 5 harboring *GABRA2*, *GABRG1*, and *GABRA6* were found to be associated with ND in several independent Caucasian populations. Furthermore, the involvement of the GABAergic signaling pathway, to which these genes belong, in the etiology of ND has been confirmed by pathway-based association analysis.

In spite of this progress in molecular genetic studies of addictions, we still have a long way to go, and there are many challenges that remain to be surmounted (Ho et al. 2010; Li 2010; van der Zwaluw and Engels 2009). These challenges include (1) further identification and replication of known and unknown genes in GABAergic and other signaling pathways and functional variants (including rare variants) for various addictive disorders through high-throughput approaches such as association study and deep sequencing, (2) study of copy number variations and their impact on gene expression in GABAergic and other addiction-related signaling pathways, (3) better understanding of the mechanisms underlying addictions at the molecular and cellular levels using both in vitro and in vivo approaches, and (4) determining appropriate ways to define environmental factors such that we can assess how gene–environment interaction affects addictions. An improvement of our understanding of the genetic and environmental factors underlying drug addiction has considerable potential to reduce morbidity and death by revealing the most suitable methods for prevention and novel medications for treating different addictive disorders.

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Chapter 8
Contribution of Variants in *DRD2/ANKK1* on Chromosome 11 with Smoking and Other Addictions

Abstract Both nicotine and alcohol addictions are severe public health hazards worldwide. Various twin and family studies have demonstrated that genetic factors contribute to vulnerability to these addictions; however, the susceptibility genes and the variants underlying them remain largely unknown. Of the susceptibility genes investigated, *DRD2* has received much attention. Considering new evidence supporting the association of *DRD2* and its adjacent gene *ANKK1* with various addictions, in this chapter, we provide an updated view of the involvement of variants in *DRD2* and *ANKK1* in the etiology of nicotine dependence (ND) and alcohol dependence (AD) based on linkage, association, and molecular studies. These findings show that both genes are significantly associated with addictions, with the association with *ANKK1* appearing to be stronger than that with *DRD2*. More replication studies in independent samples and functional studies of some of these variants are warranted.

Keywords *ANKK1* · *DRD2* · Nicotine dependence · Alcohol dependence · Linkage analysis · Functional study · SNP · *NCAM1–TTC12–ANKK1–DRD2* · *Taq1* polymorphism

1 Introduction

Drug addictions are common brain disorders that are extremely harmful to the individual and society. Data from the World Health Organization showed 2 billion alcohol abusers, 1.3 billion tobacco users, and 230 million illicit drug users worldwide in 2004 (WHO 2008). Presumably, a large percentage of these users are dependent on their drug of choice, and a great number of AD individuals are also dependent on nicotine and vice versa. Family, twin, and adoption studies have shown a moderate heritability for both ND and AD. Estimates of the heritability of ND range from 54.6% to 69% (Hamilton et al. 2006; Hardie et al. 2006; Sullivan and Kendler 1999a, b; True et al. 1997). In a meta-analysis of 17 twin studies (Li et al. b), we obtained a weighted mean heritability for ND of 59% in male smokers and 46% in female smokers (average 56% for all smokers). Additional studies (Hamilton et al. 2006; Hardie et al. 2006; Li 2003; Sullivan and Kendler 1999)

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have revealed a similar degree of heritability across other smoking-related behaviors, including initiation and cessation. Similarly, approximately 50–64% of the population variation in AD is accounted for by genetic factors (Heath et al. 1997; McGue 1999). Both ND and AD are complex traits that are influenced by the combined effects of multiple genes, with a small effect for each gene, the environment, and interactions between genes and the environment (Edenberg and Foroud 2006; Ho et al. 2010; Li et al. b; Sullivan and Kendler 1999; Swan et al. 2003).

The dopaminergic reward system in the brain plays a critical role in substance abuse and dependence, as well as in other neuropsychiatric disorders. In particular, the dopaminergic mesocorticolimbic reward pathways have frequently been implicated in the etiology of drug addictions and other psychiatric disorders. Drugs of abuse, such as nicotine, take advantage of this system by increasing extracellular dopamine to concentrations that are higher than those triggered by natural rewards, such as food and sexual intercourse (Di Chiara et al. 2004; Little 2000). This reward system consists of three parts: dopamine receptors, transporters, and enzyme targets. The dopamine transporters (DATs) and presynaptic and postsynaptic receptors jointly modulate the synaptic concentrations of dopamine. Whereas the magnitude and duration of dopaminergic transmission are influenced by DATs, the D2 prereceptor inhibits the rate-limiting enzyme of dopamine synthesis. A large number of studies (Sokoloff et al. 1990; Sunahara et al. 1991; Tiberi et al. 1991; Van Tol et al. 1991) have indicated two subfamilies of dopamine receptors (D1-like, including D1 and D5, and D2-like, including D2, D3, and D4) with different properties and functions. Of the five dopamine receptors, D1 (D1a and D1b) and D2 are two components of the dopaminergic system. Consequently, numerous studies have focused on determining whether variants in *DRD1* or *DRD2* could explain the heritable variation in susceptibility to addiction.

The dopamine D2 receptor, encoded by *DRD2*, is coupled to G_i-inhibitory G-proteins and generally reduces the formation of intracellular cAMP when activated. The D2 receptors are widely expressed in the human brain, with two main splice isoforms: D2 long (D2L) and D2 short (D2S; lacking exon 6). Using a rodent D2L knockout model, the functions of these two isoforms have been characterized. The D2L isoform is expressed postsynaptically, whereas the D2S isoform activity is observed mainly presynaptically (Zhang et al. 2007). Experimentally, presynaptic and postsynaptic D2S receptors inhibit dopamine release and D1 receptor responses, respectively (Rouge-Pont et al. 2002; Usiello et al. 2000). Further, D2L receptors are targeted by dopamine antagonists such as haloperidol and work in synergy with D1 receptors (Usiello et al. 2000). Because the dopamine D2 receptor is a vital part of the dopaminergic system, variants in *DRD2*, especially those functional ones, represent plausible candidates for genetic contributors to drug dependence and other psychiatric disorders.

A kinase, ankyrin repeat and kinase domain containing 1, encoded by *ANKK1*, is adjacent to *DRD2* on chromosome 11 in the human genome (Neville et al. 2004). *ANKK1*, also known as protein kinase *PKK2* or sugen kinase 288 (*SgK288*), contains a single serine/threonine kinase domain and 11 ankyrin repeats and is a member of a protein family involved in signal transduction. The ANKK1 protein is

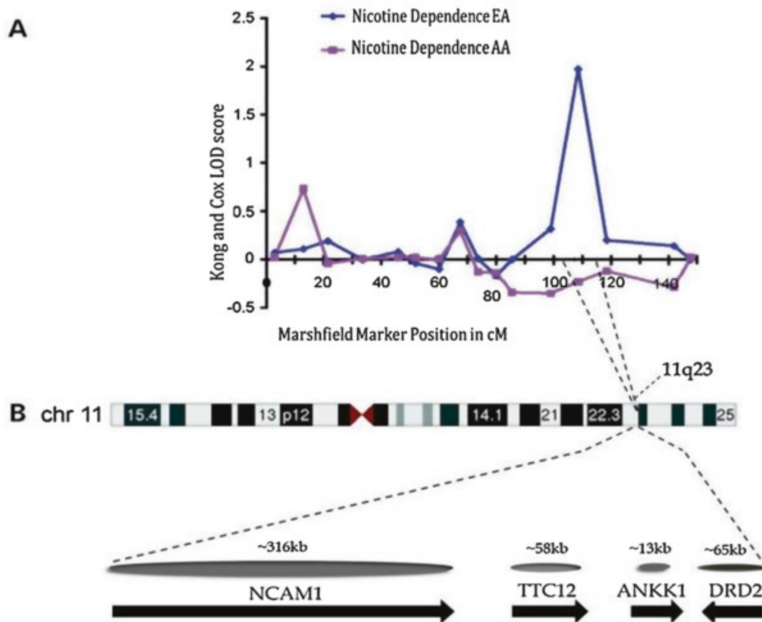

Fig. 8.1 Linkage analysis results of ND and mapping of the *NCAM1–TTC12 –ANKK1–DRD2* region on chromosome 11. (**a**) Two-point linkage analysis plot for FTND on chromosome 11 (Gelernter et al. 2007). (**b**) Location of gene cluster *NCAM1–TTC12–ANKK1–DRD2* on chromosome 11. Sizes of *NCAM1*, *TTC12*, *ANKK1*, and *DRD2* are about 316 kb, 58 kb, 13 kb, and 65 kb, respectively (Yang et al. 2007) (Reprinted from Gelernter et al. 2006 with permission from Oxford University Press)

suggested to be involved in dopaminergic reward processes via signal transduction or other cellular effects. Thus, it has been hypothesized that variants in *ANKK1* are involved in the etiology of addiction. The neural cell adhesion molecule (*NCAM1*) locus, located close to *DRD2*, has also been considered a candidate for a gene influencing addiction. *ANKK1* and another gene called tetratricopeptide repeat domain 12 (*TTC12*) are located between *DRD2* and *NCAM1*. Because functionally related genes tend to be clustered (Neville et al. 2004), it is possible that these genes close to *DRD2* also are involved in dopaminergic reward processes. Thus, this gene cluster, *NCAM1–TTC12–ANKK1–DRD2* (Fig. 8.1), is presumed to be associated with addictions. Indeed, previous linkage studies of smoking behavior have shown a significant linkage signal for this gene cluster (Gelernter et al. 2007; Morley et al. 2006). Further, many genetic association studies (Dick et al. 2007b; Gelernter et al. 2006; Nelson et al. 2013; Yang et al. 2007) suggest involvement of this gene cluster, especially *DRD2/ANKK1*, in addictions.

The primary objective of this chapter is to provide an updated view of recent studies on the significant association of *DRD2/ANKK1* with nicotine and alcohol dependence. Then we focus on the present evidence for functional SNPs in *DRD2/*

ANKK1 that has been gained from molecular studies to understand how genetic factors confer susceptibility to addictions.

2 Evidence from Genome-Wide Linkage Analysis of Involvement of *DRD2/ANKK1* in Addictions

During the past dozen years, a large number of genome-wide linkage studies have been conducted for various addiction-related phenotypes. Regions on chromosomes 4, 5, 9–11, and 17 were found to be more likely to harbor risk genes for addiction to multiple substances (Chaps. 4 and 10). Of them, the linkage regions on chromosome 11 have been significant in several studies of different addictive phenotypes. For example, Li and his coworkers (Li et al. 2003a) identified a significant linkage to smoking behavior on chromosome 11q12 (logarithm of the odds [LOD] score 3.95), and Bierut et al. (2004) found a linkage to chromosome 11q14 for habitual smoking (HS) and HS with comorbid AD.

A small but growing number of studies on smoking behavior also observed a linkage signal close to *DRD2*. Morley et al. (2006) explored the evidence for sex differences in smoking initiation and cigarette consumption in an Australian twin sample and incorporated sex differences into linkage analyses for these phenotypes using the rigorous criteria proposed by Lander and Kruglyak (1995). Those investigators observed the highest peak ($P = 0.00399$) for cigarette consumption on chromosome 11q23. Replication of the finding reached the threshold for significance ($P = 0.01$) on 11q23–24. Further, Gelernter and colleagues (2007) demonstrated a relatively significant linkage of a region on chromosome 11 to ND in a European American (EA) sample (LOD score 1.97 at 108.59 cM near marker D11S908; see Fig. 8.1). Because this linked region for ND is in proximity to the candidate gene cluster *NCAM1–TTC12–ANKK1–DRD2*, the genes included in this cluster have been suggested to contribute to the detected linkage signal, although direct evidence of such a contribution is lacking.

3 Evidence from Candidate Gene-Based Association Studies

Experimental data have indicated that *DRD2* is a susceptibility gene for smoking behaviors. Although many studies have investigated the association between *DRD2* and cigarette smoking, only a limited number of variants showed a significant association (Table 8.1). Previously, studies were concentrated mainly on the *Taq1A* polymorphism (i.e., dbSNP rs1800497). Both *in vivo* and other studies indicate that *Taq1A* is associated with reduced dopamine D2 receptor densities and binding affinity (Jonsson et al. 1999a; Noble 2003; Noble et al. 1991; Pohjalainen et al. 1998; Thompson et al. 1997). It has been suggested that the *Taq1A* variant directly or

Table 8.1 Replicated SNPs in *DRD2/ANKK1* association with various addictive phenotypes

Gene	dbSNP ID	Chr. position	Phenotype	Ethnicity	Associated risk allele/genotype	Reported P value	References
DRD2	rs6589377	113355736	ND	AA and Han Chinese	NR	0.0001	Huang et al. (2009) and Wei et al. (2012)
	rs1799978	112851561	AD	Caucasian	NR	0.02	Dick et al. (2007b)
				EA and AA	G	0.04	Yan et al. (2013)
			HD	Han Chinese	GG	0.042	Xu et al. (2004)
	rs1799732	112851462	ND	EA and AA	NR	0.002	Gelernter et al. (2006)
			AD	MA, Japanese, and Indian (male)	-141C Ins	<0.002	Du and Wan (2009), Ishiguro et al. (1998), Konishi et al. (2004), Prasad et al. (2010)
			HD	Han Chinese	-141C Ins	0.002	Li et al. (2002) and Xu et al. (2004)
			OD	Arabian	-141C Del	0.000026	Al-Eitan et al. (2012)
	rs4648318	112818599	ND	EA and AA	NR	0.041	Huang et al. (2009)
				Finnish	G	0.001	Ducci et al. (2011)
	rs1079597	112801496	ND	EA and AA	NR	0.003	Gelernter et al. (2006)
				Caucasian	A	0.048	De Ruyck et al. (2010) and Spitz et al. (1998)
			AD	Caucasian	A	0.008	Blum et al. (1993)
			HD	Hungarian and Han Chinese	A	0.00017	Vereczkei et al. (2013) and Xu et al. (2004)
			CD	Caucasian	A	0.006	Noble et al. (1993) and Persico et al. (1996)
	rs1800498	112796798	ND	EA and AA	NR	0.004	Gelernter et al. (2006)
			HD	Han Chinese	G	0.01	Xu et al. (2004)
	rs2283265	112790746	CD	EA, AA, and other	T	0.001	Moyer et al. (2011) and Sullivan et al. 2013)
			HD	Arabian	T	0.001	Al-Eitan et al. (2012)

(continued)

Table 8.1 (continued)

Gene	dbSNP ID	Chr. position	Phenotype	Ethnicity	Associated risk allele/genotype	Reported P value	References
	rs1076560	112788898	Smoking	EA, AA, and other	T	0.008	Morton et al. (2006)
			AD	Japanese (male)	A	0.034	Sasabe et al. (2007)
			CD	EA, AA, and other	T	0.001	Moyer et al. (2011) and Sullivan et al. 2013)
			HD	Arabian	T	0.031	Al-Eitan et al. (2012)
			OD	Caucasian	T	0.022	Doehring et al. (2009)
				EA	A	0.03	Toni-Kim Clarke et al. (2013)
				AA	A	0.02	Toni-Kim Clarke et al. (2013)
				Pooled sample (EA + AA)	A	0.0038	Toni-Kim Clarke et al. (2013)
	rs6277	112788669	ND	EA and AA	NR	0.001	Gelernter et al. (2006)
				Caucasian	T	0.006	De Ruyck et al. (2010)
				Caucasian	C	0.022	Voisey et al. (2012)
			Smoking	EA, AA, and other	C	0.02	Morton et al. (2006)
			AD	Caucasian	T	0.038	Hill et al. (2008)
				Australian	C	0.022	Swagell et al. (2012)

ANKK1	rs1800497	112776038	ND	EA, AA, and other	A1	<0.001 (P-meta)	De Ruyck et al. (2010), Huang et al. (2009), Li et al. (2004), and Voisey et al. (2012)
			AD	Caucasian and other	A1	<0.001	Amadeo et al. (1993), Berggren et al. (2006), Blum et al. (1990), Blum et al. (1993), Comings et al. (1991), Ishiguro et al. (1998), Ovchinnikov et al. (1999), Prasad et al. (2010), and Preuss et al. (2007)
			HD	Hungarian and Han Chinese	A1	0.009	Hou and Li (2009) and Vereczkei et al. (2013)
			CD	Caucasian	A1	0.00001	Noble et al. (1993) and Persico et al. 1996)
	rs2734849	112775370	ND	EA and AA	A	0.00053	Huang et al. (2009)
				Finnish	A	0.0002	Ducci et al. (2011)
			AD and MC	Caucasian	NR	0.02	Dick et al. (2007b)
			AD andASPD	Caucasian	NR	0.04	Dick et al. (2007b)
	rs4938016	112775225	ND	EA and AA	NR	0.002	Gelernter et al. (2006)
			AD	Caucasian	NR	0.03	Dick et al. (2007b)
	rs11604671	112773269	ND	EA and AA	A	0.0091	Huang et al. (2009)
				EA and AA	NR	0.0007	Gelernter et al. (2006)
			AD &ASPD	Caucasian	NR	0.03	Dick et al. (2007b)
	rs4938015	112769854	ND	EA and AA	NR	0.00009	Gelernter et al. (2006)
			AD and MC	Caucasian	NR	0.02	Dick et al. (2007b)

(continued)

Table 8.1 (continued)

Gene	dbSNP ID	Chr. position	Phenotype	Ethnicity	Associated risk allele/genotype	Reported P value	References
	rs4938013	112769680	ND	EA and AA	NR	0.00003	Gelernter et al. (2006)
			HD	Australian	NR	0.0000013	Nelson et al. (2013)
	rs17115439	112769482	AD	Caucasian	NR	0.04	Dick et al. (2007b)
				EA and AA	C	0.077	Yan et al. (2013)
	rs4938012	112764864	ND	EA and AA	NR	0.000008	Gelernter et al. (2006)
			AD	Caucasian	NR	0.03	Dick et al. (2007b)
			AD + MC	Caucasian	NR	0.008	Dick et al. (2007b)
	rs877138	112761718	ND	Finnish	A	0.001	Ducci et al. (2011)
			HD	Australian	NR	0.000001	Nelson et al. (2013)
			AD + MC	Caucasian	NR	0.03	Dick et al. (2007b)
			AD + ASPD	Caucasian	NR	0.02	Dick et al. (2007b)
	rs2282511	112749387	ND	EA and AA	NR	0.00009	Gelernter et al. (2006)
			AD + MC	Caucasian	NR	0.009	Dick et al. (2007b)
			AD + ASPD	Caucasian	NR	0.03	Dick et al. (2007b)

(1) Information of significant SNPs across *DRD2/ANKK1* replicated by at least two independent studies; (2) *NR* not reported; (3) *Reported P value* minimum P value from related paper(s); (4) *EA* European American, *AA* African-American, *MA* Mexican-American; (5) *ND* nicotine dependence, *AD* alcohol dependence, *AD + MC* alcohol dependence + medical complications (assessed by an item in the SSAGA that asked if the subject had ever experienced any of the following health problems as a result of prolonged use of alcohol: liver disease or yellow jaundice, stomach disease or vomiting blood, pancreatitis, or cardiomyopathy), *AD + ASPD* alcohol dependence + comorbid antisocial personality disorders, *HD* heroin dependence, *OD* opioid or opiate dependence, *CD* cocaine dependence

indirectly influences the concentration of dopamine in the synaptic clefts. Noble et al. (1994) first showed that former and current smokers have a significantly higher prevalence of the A1 allele than non-smokers, and a subsequent study replicated this finding (Comings et al. 1996a). Further, genetic association studies (De Ruyck et al. 2010; Voisey et al. 2012) demonstrated that the *Taq1A* polymorphism was significantly associated with ND. Accruing pharmacogenetic studies (Cinciripini et al. 2004; Stapleton et al. 2011; Wilcox et al. 2011) also indicate an association between the polymorphism of *Taq1A* and smoking cessation. A meta-analysis of 12 studies (Li et al. 2004) demonstrated a significantly higher prevalence of the *Taq1A* A1 allele in smokers than in non-smokers ($P < 0.0001$; pooled odds ratio [OR] 1.50; 95% confidence interval [CI] 1.33, 1.70). On the other hand, contradictory findings have been reported (Berlin et al. 2005; Hamajima et al. 2002; Munafo et al. 2004, 2009; Yoshida et al. 2001).

In 2004, Neville and colleagues (Neville et al. 2004) first reported the identification of the *ANKK1* gene near *DRD2*. Through signal transduction or other biochemical pathways, *ANKK1* has been suggested to be involved in the dopaminergic reward system. The authors also localized the polymorphism *Taq1A* to exon 8 of *ANKK1*. Besides, *Taq1A* was demonstrated to cause a glutamate-to-lysine substitution at amino acid residue 713 in the putative binding domain of *ANKK1*. Zhang et al. (2007) reported that *Taq1A* was in strong linkage disequilibrium (LD) with two intronic single nucleotide polymorphisms (SNPs), rs2283265 and rs1076560, of *DRD2* ($D' = 0.855$). Those two SNPs have been associated with addiction and neuropsychiatric disorders such as cocaine dependence (CD) (Moyer et al. 2011) and schizophrenia (Zheng et al. 2012), as well as with a reduced density of D2S relative to D2L and D2 receptors (Zhang et al. 2007). Besides, Gelernter and colleagues (Gelernter et al. 2006) showed that *Taq1A* is in LD with two functional variants of *ANKK1* ($D' = 0.73$ with rs4938015 and $D' = 1.0$ with rs11604671), and these two SNPs have been associated with ND (Gelernter et al. 2006) and smoking initiation and cessation (David et al. 2010). Although *Taq1A* is a non-synonymous base substitution, we speculate that it is perhaps a proxy in LD with a causative variant(s) in *DRD2* or *ANKK1*, providing some explanation for heterogeneities in the results of various studies.

After the identification of *ANKK1*, many researchers in the drug addiction field have turned their attention to variants in both *DRD2* and *ANKK1*. Several family-based association studies demonstrated that variants spanning *DRD2* and *ANKK1* have a significant association with ND; variants in *ANKK1* display a stronger association signal. By genotyping a set of 43 SNPs spanning the *NCAM1–TTC12–ANKK1–DRD2* gene cluster in 1615 participants from 632 families (319 African-American [AA] and 313 EA), Gelernter et al. (2006) performed family-based association and haplotype analysis to explore causative variants significantly associated with ND. They revealed that SNPs in *DRD2* and *NCAM1* showed weak evidence of association with ND, but SNPs in *TTC12* and *ANKK1* had strong evidence of association with ND. For *ANKK1*, four statistically significantly associated SNPs were found: rs4938012, rs4938013, rs4938015, and rs11604671. Of them, rs4938012 showed the most significant association with ND in the combined sample

($P = 0.000008$). The haplotype G-A-T-C, formed by SNPs rs2303380–rs4938012–rs4938015–rs11604671 spanning *TTC12/ANKK1*, showed the most significant association with ND in the combined sample ($P = 0.0000001$). Those investigators also revealed that the haplotypes A-G-C-T and A-G-T-C, formed by the same four SNPs, are significantly associated with a reduced risk of ND in both EAs ($P = 0.001$) and AAs ($P = 0.0009$).

We did a similar association analysis in the Mid-South Tobacco Family (MSTF) cohort, including 2037 subjects in 602 nuclear families (671 subjects from 200 EA families and 1366 subjects from 402 AA families; Huang et al. 2009). They selected 16 SNPs across *DRD2* and 7 SNPs across *ANKK1* and applied three common measures to ascertain the degree of ND, i.e., smoking quantity (SQ), the Heaviness of Smoking Index (HSI), and the Fagerström Test for ND (FTND). The polymorphism of rs2734849 in *ANKK1* showed a significant association ($P = 0.00053$–0.010) with all three ND measures in both the AA and the pooled samples. However, after correction for multiple testing, variants in *DRD2* showed only a weak association. In addition, by using the luciferase reporter assay, we demonstrated the polymorphism rs2734849 to be associated with altered expression of NF-κB-regulated genes that might indirectly affect *DRD2* density. Ducci et al. (2011) also reported that rs2734849 was significantly associated with smoking ($P = 0.0002$) in adolescent subjects.

Recently, a large population-based study (Eicher et al. 2013) examined the risk factor of language impairment (LI) and reading disability. High prenatal nicotine exposure increased LI risk in a dose–response manner (OR 3.84; $P = 0.0002$). Next, the authors investigated the association of variants in genes involved in nicotine-related pathways, which revealed significant associations between *DRD2/ANKK1* variants and performance on language tasks. The finding of a significant association of SNPs in *ANKK1* with LI was replicated in an independent case-control study ($P < 0.05$). These findings not only indicate that *DRD2/ANKK1* play a significant role in nicotine-related pathways and dopamine signaling involved in language processing but also provide evidence for involvement of variants in *DRD2/ANKK1* in addictions and other psychiatric disorders.

Taken together, the data from genome-wide linkage studies have indicated that *DRD2* is a risk gene for smoking-related behaviors. Given prior knowledge, a great number of genetic association studies have been focused on the relation between *DRD2* and smoking behaviors. Although inconsistent results remain to be explained, a certain number of variants in *DRD2* have been significantly associated with ND and other smoking-related behaviors. As converging evidence accumulated, this implies a critical role for *DRD2* in the process of ND. Several family-based association studies with large samples also showed a prominent association between *ANKK1* and ND, and the association signal of ND with variants in *ANKK1* appears to be stronger than that for *DRD2*. Further, the product of *ANKK1* is apparently involved in the dopaminergic reward system. Thus, *ANKK1* should be regarded as a susceptibility gene for ND and related smoking behaviors. However, the biological mechanism underlying the involvement of variants in *DRD2/ANKK1* in ND remains to be characterized.

4 Studies on Alcohol Dependence

Similarly, accumulating studies have focused on whether there exists a significant association between *DRD2/ANKK1* and alcoholism. Wise and Rompre (1989) showed that the rewarding effect of alcohol is mediated through mesolimbic dopamine. Many experimental studies have since revealed that reduced concentrations of *DRD2* increase alcohol intake (Heinz et al. 2004; Tupala et al. 2003; Volkow et al. 1996), whereas overexpression of *DRD2* reduces alcohol intake (Thanos et al. 2001, 2004, 2005). A large number of genetic association studies also indicate a positive association (Table 8.1).

As described above, the polymorphism *Taq1A* has been widely studied in relation to smoking behaviors. It also was examined in relation to other addictions or mental disorders, such as opioid dependence, schizophrenia, and, in particular, AD. After Blum et al. (1990) first reported an association between the *Taq1A* polymorphism and alcoholism, follow-up studies (Amadeo et al. 1993; Berggren et al. 2006; Comings et al. 1991; Ovchinnikov et al. 1999) provided independent replication. Nevertheless, conflicting findings have been reported (Anghelescu et al. 2001; Gelernter et al. 1991; Sander et al. 1999). Of note, most positive findings resulted from studies on European or EA samples, whereas studies in other ethnic samples generally have been negative (Chen et al. 1996; Gelernter and Kranzler 1999; Lee et al. 1997; Lu et al. 1996). Four meta-analyses (Le Foll et al. 2009; Munafo et al. 2007; Smith et al. 2008; Wang et al. 2013) demonstrated *Taq1A* to be a risk variant for AD and also showed significant heterogeneity between studies. A number of genetic association studies have concentrated on the association between other variants in *DRD2* and AD (Du and Wan 2009; Hill et al. 2008; Konishi et al. 2004; Swagell et al. 2012). In particular, C957T, *Taq1B*, and -141C *Ins/Del* have been extensively investigated, although the results remain equivocal. However, a growing number of genetic association studies show a strong association signal of AD derived from *ANKK1* but not *DRD2* (Dick et al. 2007b; Yang et al. 2007), a finding similar to that with ND.

After Gelernter et al. (2006) reported a significant association of *TTC12* and *ANKK1* with ND, Yang et al. (2007) observed a consistent result with AD in 1220 EA participants using family-based (n = 488 subjects) and case-control (n = 318 cases and 414 controls) analyses. Through two association studies for 43 SNPs spanning the *NCAM1–TTC12–ANKK1–DRD2* gene cluster, Yang et al. (2007) implicated variants in exons 2 and 5 of *ANKK1* as prominent risk factors for AD. Further, by selecting 26 SNPs spanning *DRD2* and *ANKK1* (16 SNPs across *DRD2* and 10 across *ANKK1*) in a sample of 1923 participants representing 219 Caucasian families from the Collaborative Study on the Genetics of Alcoholism (COGA), Dick and coworkers (Dick et al. 2007b) analyzed this region for AD based on characterizing the AD phenotype (AD, AD + medical complications, and AD + antisocial personality disorders [ASPD]). They found that SNPs located in *ANKK1* are significantly associated with the AD phenotype; in particular, the polymorphism rs4938012 in the 5′ LD block of *ANKK1* showed the most significant association

($P = 0.008$) with AD + medical complications. For *DRD2*, only a small number of SNPs showed weak associations with AD.

Most recently, Nelson et al. (2013) examined 71 SNPs in the *NCAM1–TTC12–ANKK1–DRD2* gene cluster in 3485 Australian subjects to perform a case-control-based genetic association study on heroin dependence (HD). In this study, there was one case sample selected from opioid replacement therapy clinics ($n = 1459$) and two control samples: one from economically disadvantaged areas near the clinics ($n = 531$) and another from 1459 unrelated Australians without dependence on alcohol or illicit drugs who were enrolled in a twin and family study sample. Those investigators showed a significant association between *ANKK1* and illicit drug dependence, but none of the SNPs in *DRD2* showed a significant association. Comparing case samples ($n = 1459$) with the subgroup of neighborhood controls not dependent on illicit drugs ($n = 340$), two SNPs (rs877138 and rs4938013) in *ANKK1* showed a significant association with HD after correcting for multiple testing. In particular, the SNP rs877138 showed the strongest significant association ($P = 9.7 \times 10^{-7}$; OR 1.59; 95% CI 1.32, 1.92) with HD. In the comparison between individuals with and without a lifetime history of illicit drug dependence in the neighborhood control group, rs877138 was significantly associated with HD ($P = 8.0 \times 10^{-4}$), suggesting this SNP is also a risk factor for other illicit drug dependences. Taken together, these studies show that the association between the variants in *ANKK1* and AD is not only significant but also stronger than that of *DRD2*. This association pattern was found in other addiction studies as well (Nelson et al. 2013). In sum, these findings confirm that *ANKK1* is a plausible candidate gene for an encouragement of addiction.

In view of the crucial function of *DRD2* in the human dopaminergic reward system, current evidence from genetic association studies on its link to addiction is unsatisfactory. One possible contributor to this problem is the inconsistent definition of addiction-related phenotypes in various studies. A recent study performed by Meyers and coworkers (Meyers et al. 2013), which examined 28 SNPs across *DRD2* and 3 SNPs across *ANKK1* in a subset of the population-based Finnish twin sample ($n = 602$), revealed a significant association of the SNPs rs10891549, rs1554929, rs6275, and rs6279 in *DRD2* with alcohol problems. Additionally, by examining the association between *DRD2* and various alcohol phenotypes, Connor et al. (2002) found that *DRD2* was associated with alcohol consumption quantity, the amount of alcohol consumed per week, and AD but not with the frequency of alcohol use. Although there exists a strong genetic correlation among different alcohol phenotypes (Grant et al. 2009; Kendler et al. 2010), available evidence suggests that these phenotypes are controlled by different genetic variants and alleles (Dick et al. 2011). Thus, it is important in a future study to focus on the variability in the measurement of alcohol phenotypes in order to identify more causative variants across *DRD2/ANKK1*.

5 Comorbidity of Substance Dependence

As is clear from the literature, various addiction-related phenotypes tend to occur together (Bien and Burge 1990; Collins 1990; Dani and Harris 2005; Darke and Hall 1995; Darke and Ross 1997; Dinwiddie et al. 1996; John et al. 2003; Zacny 1990). Drugs of abuse often take advantage of similar pathways to increase extracellular dopamine in the brain. Blomqvist and colleagues (1997) showed that ethanol enhances locomotor activity and dopamine release can be antagonized by the nicotinic channel blocker mecamylamine in animal models. Commonly, drug abusers take multiple drugs. For example, primary heroin users also take cannabis, benzodiazepines, alcohol, and amphetamines (Kidorf et al. 1996; Klee et al. 1990), and 63% of methadone maintenance patients have AD, while 50% have benzodiazepine dependence.

Many studies also have suggested a high correlation between alcohol consumption and cigarette smoking (Bien and Burge 1990; Collins 1990; Zacny 1990). For example, a twin study (True et al. 1999) revealed a genetic correlation of 0.68 (95% CI 0.61, 0.74). Heavy smokers are known to be predisposed to be heavy drinkers (Breslau 1995; Preuss et al. 2007). A large number of independent genetic association studies (Du and Wan 2009; Gelernter et al. 2006; Hill et al. 2008; Konishi et al. 2004; Swagell et al. 2012; Voisey et al. 2012) have reported those two substances to have numerous common genetic variations in *DRD2/ANKK1* that are associated with a risk of dependence, e.g., C957T, -141C *Ins/Del*, and *Taq1A* (see Table 8.1). In addition, other substance dependences influence the association of genetic factors with AD or ND (Agrawal et al. 2006; Dick et al. 2007a; Yang et al. 2008). For example, Yang et al. (2008) conducted association studies of AD + DD and AD without DD in 1090 EAs using both family-based and case-control-based designs on comorbid alcohol and drug dependencies. They found that variants at the 3′ ends of *ANKK1* and *DRD2* regulate the risk of AD, with effects depending on comorbidity with DD.

For decades, motor, cognitive, emotional, and social deficits have been considered markers of human disorders such as compulsive drug use, schizophrenia, and Parkinson's disease. Those syndromes also have been related to the sensitivity of *DRD2*. Many variations in *DRD2/ANKK1* have been associated with higher risks of impulsive traits or mental disorders (Table 8.2). Thus, those significant variants probably confer susceptibility to AD, ND, or both. For example, a number of independent studies found a polymorphism of C957T to be significantly associated with psychiatric disorders, e.g., schizophrenia (Hanninen et al. 2006; Lawford et al. 2005) and personality disorder (Perkins et al. 2008; Ponce et al. 2008), as well as ND (Gelernter et al. 2006) and AD (Hill et al. 2008). This indicates that significant variants observed in other psychiatric disorders or addictive behaviors may contribute to causative variants for AD or ND.

Table 8.2 Example of significant SNPs in *DRD2/ANKK1* association with psychiatric and neurologic phenotype

Gene	dbSNP ID	Phenotype	Population	References
DRD2 *DRD2*	rs1799978	Childhood aggression	Caucasian- and African-Canadians and mixed ethnicity	Zai et al. (2012)
		Antipsychotic treatment response in first-episode schizophrenic patients	AA, Caucasian, Hispanic, and mixed ethnicity	Lencz et al. (2006)
		Clozapine treatment response in schizophrenic patients	South Han Chinese	Gong et al. (2011)
		Schizophrenia	Japanese	Ikeda et al. (2008)
	rs1799732	Human maternal behavior	Caucasian	Mileva-Seitz et al. (2012)
		BPAD	Han Chinese	Li et al. (1999)
		Earlier clinical presentation of Wilson disease neuropsychiatric symptoms	Polish	Litwin et al. (2013)
		Neuroleptic malignant syndrome	Japanese	Kishida et al. (2004)
		Antipsychotic-induced weight gain in schizophrenia	Caucasian and non-Caucasian	Lencz et al. (2010)
		Poorer antipsychotic drug response in schizophrenic patients	Meta-analysis including mixed ethnicity	Zhang et al. (2010)
		Antipsychotic treatment response in schizophrenic patients	African-American, Caucasian, Hispanic, and mixed ethnicity	Lencz et al. (2006)
		Schizophrenia	Brazilian	Cordeiro et al. (2009)
		Schizophrenia	Japanese	Arinami et al. (1997), Inada et al. 1999), and Ohara et al. (1998)
		Schizophrenia	Caucasian	Breen et al. (1999) and Jonsson et al. (1999b)

(continued)

Table 8.2 (continued)

Gene	dbSNP ID	Phenotype	Population	References
	rs1079597	Borderline personality traits	US young adult (mixed ethnicity)	Nemoda et al. (2010)
		Parkinson's disease	European	Oliveri et al. (2000)
		Tourette's syndrome	Antioquian	Herzberg et al. (2010)
		Clozapine treatment response in schizophrenic patients	African-American	Hwang et al. (2005)
		Severe hallucination in schizophrenic patients	South Indian	Vijayan et al. (2007)
		Schizophrenia	European	Dubertret et al. (2001)
	rs1800498	Autism spectrum disorders	Caucasian and others	Hettinger et al. (2012)
		Parkinson's disease	South Indian	Juyal et al. (2006)
		Treatment response in schizophrenia, suspiciousness, hallucination, and bizarre behavior	South Indian	Vijayan et al. (2007)
	rs2283265	Binge eating disorder	Caucasian and mixed ethnicity	Davis et al. (2012)
		Reduced performance in working memory and attentional control tasks in healthy humans	Caucasian	Zhang et al. (2007)
		Severe negative symptoms in schizophrenic patients	Han Chinese	Chien et al. (2013)
		Schizophrenia	Han Chinese	Glatt et al. (2009)
	rs1076560	Emotional processing	NR	Blasi et al. (2009)
		Reduced performance in working memory and attentional control tasks in healthy subjects	Caucasian	Zhang et al. (2007)
		Cingulate response during attentional control and behavioral accuracy during sustained attention in health subjects and response to 8 weeks of treatment with olanzapine in schizophrenia	European	Blasi et al. (2011)
		Schizophrenia	Han Chinese	Zheng et al. (2012)
	rs6277	Binge eating disorder	Caucasian and mixed ethnicity	Davis et al. (2012)
		Stuttering	Han Chinese	Lan et al. (2009)

(continued)

Table 8.2 (continued)

Gene	dbSNP ID	Phenotype	Population	References
		Human maternal behavior	Caucasian	Mileva-Seitz et al. (2012)
		Negative feedback learning	Caucasian	Frank and Hutchison (2009) and Frank et al. 2007)
		Motor learning	Caucasian	Huertas et al. (2012)
		Working memory	Caucasian	Xu et al. (2007)
		Confirmation bias	Caucasian, Asian, African-American, and other	Doll et al. (2011)
		Impulsivity	Caucasian and mixed ethnicity	White et al. (2009)
		Impulsivity	Japanese	Yoshiya Kawamura et al. (2013)
		Dysfunctional impulsivity	Caucasian	Colzato et al. (2010)
		Human fear conditioning and aversive priming	Spanish	Huertas et al. (2010)
		PTSD	Caucasian	Voisey et al. (2009)
		Schizophrenia	Bulgarian	Betcheva et al. (2009)
		Schizophrenia	Han Chinese	Fan et al. (2010)
		Schizophrenia	Finnish	Hanninen et al. (2006)
		Schizophrenia	Spanish	Hoenicka et al. (2006)
		Schizophrenia	Caucasian	Lawford et al. (2005)
ANKK1	rs1800497	Binge eating disorder	Caucasian and mixed ethnicity	Davis et al. (2008), Davis et al. (2012), and Nisoli et al. (2007)
		ADHD	Caucasian	Comings et al. (1991) and Sery et al. (2006)
		Avoidance learning	Caucasian	Frank and Hutchison (2009) and Klein et al. (2007)

(continued)

Table 8.2 (continued)

Gene	dbSNP ID	Phenotype	Population	References
		Childhood aggression	Caucasian, African-Canadians, and mixed ethnicity	Zai et al. (2012)
		Autism spectrum disorders	Caucasian	Comings et al. (1991)
		Social alienation	NR	Hill et al. (1999)
		Antisocial personality disorder	Spanish	Ponce et al. (2003)
		Borderline personality traits	Mixed ethnicity	Nemoda et al. (2010)
		Impulsivity	Caucasian and mixed ethnicity	Dan TA Eisenberg et al. (2007) and White et al. (2008)
		BPAD	Han Chinese	Li et al. (1999)
		PTSD	Caucasian	Comings et al. (1991), Comings et al. (1996b)
		Tourette's syndrome	Caucasian	Comings et al. (1991) and Comings et al. (1996c)
		Tourette's syndrome	Taiwanese	Lee et al. (2005)
		Parkinson's disease	European	Grevle et al. (2000) and Oliveri et al. (2000)
		Parkinson's disease	European and African-American	McGuire et al. (2011)
		Short-term haloperidol treatment response in patients with acute psychosis	Caucasian	Schafer et al. (2001)
		Clozapine treatment response in schizophrenic patients	African-American	Hwang et al. (2005)
		Schizophrenia and severe self-neglect symptoms in schizophrenic patients	South Indian	Vijayan et al. (2007)
		Schizophrenia	European	Dubertret et al. (2001) and Dubertret et al. (2004)

(1) Those SNPs, associated with addiction, have been extensively investigated for psychiatric and neurologic phenotypes; (2) *BPAD* bipolar affective disorder; (3) *PTSD* post-traumatic stress disorder; (4) *ADHD* attention deficit hyperactivity disorders; (5) *NR* not reported

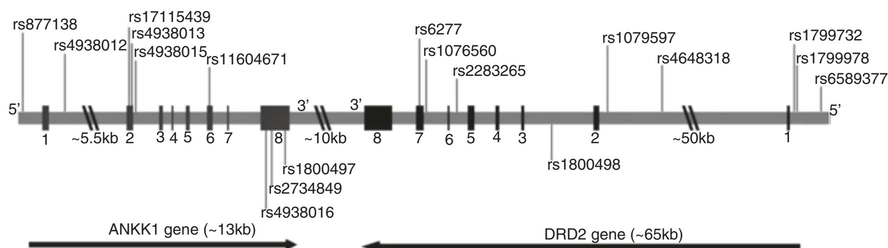

Fig. 8.2 Location of SNPs in *DRD2* and *ANKK1* replicated by at least two independent studies. Exons or 5′- and 3′-UTRs for *DRD2* are shown by *dark bars*, exons for *ANKK1* by *light black bars*, and introns for *ANKK1/DRD2* by *horizontal gray lines*. The *DRD2/ANKK1* organization is shown to its original scale, except for the part between the 3′ end of *ANKK1* and the 5′ end of *DRD2*, based on the NCBI genome contig NT_033899

6 Evidence from Molecular Studies of Functional SNPs in *DRD2/ANKK1*

Previous genetic association studies have implied that many variants of *DRD2/ANKK1* are significantly associated with addiction. Those variants have been observed in promoters, introns, and exons; and some of them have been replicated by at least two independent studies (see Table 8.1 and Fig. 8.2). However, only a few variants across *DRD2/ANKK1* are reported to be associated with altered D2 receptor-related brain function. Meanwhile, evidence for the function of *ANKK1* is absent, considering that it likely acts in the dopaminergic system. Thus, more functional studies need to be performed aiming to understand how *DRD2/ANKK1* variants exert their effects on brain biology. In this part, we focus on several functional variants across *DRD2/ANKK1* that has been investigated extensively by molecular studies.

7 Potentially Functional Genetic Variations of DRD2

Numerous studies have demonstrated that the 5′-flanking region of *DRD2* has a critical role in regulatory actions (Bontempi et al. 2007; Fiorentini et al. 2002); thus, variants in this region are likely to be involved in regulating the expression of the D2 receptor gene. In *DRD2*, the SNP rs1799732 or -141C *Ins/Del*, suggested by initial in vitro gene expression experiments, appears to be a functional variant. Arinami and coworkers (1997) examined the effect on gene expression of the *DRD2* promoter region sequence containing either the -141C *Ins* or *Del* allele. Created by means of transient transfection and luciferase assay, the –141C *Del* allele-containing construct showed only 21% of the D2 receptor gene expression in human retinoblastoma Y-79 cells and 43% in human embryonal kidney (HEK) 293 cells relative to the -141C *Ins* allele-containing construct. Moreover, using spiperone and a small

sample of postmortem brains of non-schizophrenic patients, those investigators found that, consistently, the number of spiperone-binding sites (Bmax) was reduced in the putamen of –141C *Del* allele carriers compared with non-carriers. However, an in vivo positron emission tomography (PET) study (Jonsson et al. 1999a), using [^{11}C]-raclopride and healthy volunteers, provided the opposite result, in that participants who carried the *Del* allele had a significantly higher striatal D2 receptor binding potential. In contrast, Pohjalainen et al. (1999), who performed a similar *in vivo* PET study, found no significant differences between –141C *Ins/Del* alleles in striatal D2 receptor binding potential.

Another polymorphism, rs12364283, located in the 5′-flanking region (a T/C SNP at position −844 upstream of the transcription start site), has been reported to be associated with enhanced *DRD2* expression (Zhang et al. 2007). Those researchers observed that the minor C allele of rs12364283 contributed to higher transcriptional activity than the major T allele. They also reported that two other SNPs, rs2283265 and rs1076560, located in the fifth and sixth introns of *DRD2*, showed significant association with lower expression of D2S compared with D2L and D2 receptor density. An fMRI analysis revealed a brain activity-modulating effect on working memory and attentional control tasks for both intronic variants in healthy participants. Thereafter, Bertolino et al. (2009) provided a similar result, finding that these three variants (rs12364283, rs2283265, and rs1076560) regulate schizophrenia development, possibly by modifying D2S/D2L ratios in the context of different total D2 density.

The functional synonymous variant of rs6277 or C957T, located in exon 7 of *DRD2*, is another regulator of *DRD2* expression. A previous *in vitro* study (Duan et al. 2003), utilizing CHO-K1 cells transfected with *DRD2* cDNA, found that the T allele of C957T altered the predicted mRNA folding, leading to distinctly poorer *DRD2* mRNA stability and translation, and dramatically changed dopamine-induced upregulation of *DRD2* expression. Those investigators also found that the G1101A mutation itself did not show any function as such, but 1101A co-expressed with 957T could annul the effect of the T allele on *DRD2* function. However, two in vivo studies (Hirvonen et al. 2004, 2005), using [^{11}C]-raclopride and positron emission tomography (PET), showed an inconsistent result in that the C957T SNP had a highly significant effect on D2 receptor availability (indexed by binding potential, BP_{ND}), with the lowest for the CC genotype (C/C < C/T < T/T) in the striatal region in 45 healthy subjects. A recent in vivo study (Hirvonen et al. 2009) focused on extrastriatal D2 receptor availability measured in 38 healthy male volunteers with 3D–PET and the high-affinity *DRD2* radioligand [^{11}C]-FLB457, which showed that the C/C allele of C957T was associated with high extrastriatal *DRD2* BP_{ND} throughout the cortex and the thalamus (C/C > C/T > T/T). Another study reported by the same group (Hirvonen 2009) demonstrated that the *DRD2* C957T allele prominently altered D2 receptor density in the cortex and the thalamus, whereas the same allele affected D2 receptor affinity instead of density in the striatum.

Other common variants, such as *Taq1B*, Ser311Cys, Val96Ala, and Pro310Ser, have also been associated with altered function of the D2 receptor *in vitro* and *in vivo* (Cravchik et al. 1996; Ritchie and Noble 2003). For example, the *Taq1B*

polymorphism, located in the first intron (5′ region) of *DRD2*, is involved in transcription regulation. Jönsson et al. (1999a) found the *Taq1B* allele was associated with low dopamine receptor density. However, some conflicting results have been reported (Laruelle et al. 1998). Because the growth hormone (GH) response to apomorphine administration (APD) could reflect altered function of D2 receptors (Finckh et al. 1997), the risk allele of SNP, linked with a reduced GH response to APD, is likely associated with reduced D2 receptor activity. Two studies (Finckh et al. 1997; Lucht et al. 2010) have demonstrated that SNPs rs6276 and rs1076560 in *DRD2* are significantly associated with the APD-induced GH response. Thus, it is indirectly proved that these two SNPs are associated with altered function of the D2 receptor.

8 Potentially Functional Genetic Variations of *ANKK1*

The non-synonymous SNP *Taq1A* is located in exon 8 of *ANKK1*. There have been extensive investigations of the association between *Taq1A* and addictions and other psychiatric disorders. Accumulating data show the A1 allele of *Taq1A* to be associated with altered *DRD2*-related function. Noble et al. (1991), utilizing postmortem autoradiography with tritiated spiperone as the ligand, previously observed the number of binding sites (measured by B_{max}) in 66 alcoholic or nonalcoholic subjects with the A1 allele to be significantly decreased in comparison with that of subjects with the A2 allele. Subsequently, the results of an *in vitro* study reported by Thompson et al. (1997) were consistent with the results of Noble and coworkers (1991). Further, in 1998, Pohjalainen et al. (1998), by using PET and [^{11}C]-raclopride in 54 healthy subjects, reported that the A1 allele was significantly associated with reduced *DRD2* availability. A similar in vivo PET study (Jonsson et al. 1999a) showed the allele of A1 to be associated with low *DRD2* density in 56 healthy subjects. Recently, Hirvonen (2009) further indicated that the *Taq1A* A1 allele decreased striatal, but increased cortical and thalamic, D2 receptor density.

Laakso et al. (2005) also suggested that *Taq1A* was associated with increased striatal activity of aromatic L-amino-acid decarboxylase, the final enzyme in the biosynthesis of dopamine. In some ways, presynaptic D2 receptor function probably is reflected by changed dopamine synthesis. Similar to rs6276 and rs1076560 in *DRD2*, *Taq1A* and rs11604671 were significantly associated with the APD-induced GH response ($P < 0.05$) (Lucht et al. 2010). The result indirectly showed that *Taq1A* is likely to be associated with altered function of the D2 receptor, which is consistent with the findings from PET studies (Jonsson et al. 1999a; Pohjalainen et al. 1998). It also provides a clue that rs11604671 may be a functional variant conferring risk for addiction. Meanwhile, Gelernter and colleagues (2006) reported that *Taq1A* was in complete LD with rs11604671 [D′ = 1.0].

Another non-synonymous polymorphism rs2734849, also located in exon 8 of *ANKK1*, has been reported to produce an amino acid change from arginine to

histidine in the C-terminal ankyrin repeat domain. A recent study by Huang et al. (2009) found that the SNP rs2734849 was significantly associated with ND. To further explore the function of rs2734849 in vitro, they constructed related vectors and transfected them into human neuroblastoma SH-SY5Y cells. By using the luciferase reporter assay, they observed that the rs2734849 polymorphism was significantly associated with altered expression of NF-κB-regulated genes. Because transcription factor NF-κB could modulate *DRD2* gene expression (Bontempi et al. 2007; Fiorentini et al. 2002), the authors speculated that rs2734849 may indirectly affect dopamine D2 receptor density.

Together, current studies reveal numerous functional variants that correlate with altered D2-related brain biology. One likely mechanism is that reduced D2 receptor density and availability contribute to the etiology of relevant disorders; e.g., -141C *Ins/Del*, C957T, and *Taq1B* are all associated with altered DRD2 density and availability (Arinami et al. 1997; Duan et al. 2003; Jonsson et al. 1999a). Another possible mechanism is that variants alter *DRD2* signaling by modulating alternative splicing of exon 6 to yield DRD2L and DRD2S, e.g., two intronic SNPs, rs2283265 and rs1076560 (Zhang et al. 2007). Although functional variants have been demonstrated in *DRD2*, contradictory results are available, and the number of functional variants identified is relatively small. Although cumulative molecular studies have indicated that *Taq1A* is associated with altered *DRD2* density or related functions, some questions remain to be answered, for example, how a mutation in *Taq1A* located ~9.5 kb downstream from *DRD2* could affect *DRD2* expression. One hypothesis is that *Taq1A* serves as a surrogate marker in LD with causative variant(s) within *DRD2*, e.g., rs2283265 and rs1076560 (Zhang et al. 2007).

ANKK1 has been implicated in the dopaminergic system via signal transduction or other cellular responses. Evidence from a recent study by Hoenicka et al. (2010) demonstrated that *ANKK1* mRNA and protein are expressed in the central nervous system of adult humans and rodents, being seen exclusively in astrocytes. Those investigators also reported that the amount of *ANKK1* mRNA in mouse astrocyte cultures is upregulated by the dopamine agonist apomorphine, suggesting a relation with the dopaminergic system. Besides, Garrido and coworkers (2011) observed that ANKK1 kinase is located in both the nucleus and the cytoplasm of cells, indicating nucleocytoplasmic shunting of this putative signal transducer. Further, when stimulated with apomorphine, the Ala239Thr *ANKK1*-kinase polymorphism exhibited strong expression differences in both the nucleus and the cytoplasm at the basal level. It is thus reasonable to assume that variants in *ANKK1* are implicated in the etiology of addictions. Coincidently, genetic association studies (Dick et al. 2007b; Gelernter et al. 2006; Nelson et al. 2013; Yang et al. 2007) suggest that the association between the locus 11q22-q23 and addiction is attributable to *ANKK1* variants. The mechanism of functional variants across *ANKK1* confers a risk for addiction and is likely to alter the product of *ANKK1* itself instead of *DRD2*-related function. Because the function of *ANKK1* in the dopaminergic system remains ambiguous, more research is warranted.

9 Concluding Remarks

In sum, significant progress has been made in searching for genetic variations in *DRD2/ANKK1* in relation to addictive behaviors and other psychiatric disorders. Based on the significant linkage peak for addiction that was detected on chromosome 11, where *DRD2/ANKK1* are located, and the reported associations of the two genes with various psychiatric disorders, including addiction, we conclude that variants in *DRD2/ANKK1* play an important role in the etiology of addiction. In particular, the strength of the association of *ANKK1* with addiction appears to be even greater than that for *DRD2*.

Although many positive results have been reported, the association remains controversial. A series of explanations has been published to elucidate the heterogeneous results. The first reason perhaps is variations in the definition of addiction phenotypes in different studies. Because there is a complex architecture, with many genetic factors contributing to multiple phenotypic traits (Connor et al. 2002; Dick et al. 2011; Meyers et al. 2013), using endophenotype(s) as the measure of addiction is highly encouraged because of its significant merit for such work. Further, when focusing on a specific addictive phenotype, we need to consider comorbidity as well. It has been reported that failing to screen for comorbid-related phenotypes in the control subjects being investigated by association analysis could contribute to inconsistencies in the results (Lawford et al. 1997; Neiswanger et al. 1995; Noble and Blum 1993; Noble et al. 2000). Besides, both ethnicity and sex differences are implicated as contributing to heterogeneities across studies. Because samples from different populations tend to have different allele frequencies, it is easy to see that the disparity of races in the populations examined in different studies could produce inconsistent results. For example, in contrast to numerous studies in Caucasians, in which it was found that the *Taq1A* A1 allele is significantly associated with smoking behaviors, Yoshida et al. (2001) and Hamajima et al. (2002) reported a significant association of *Taq1A* A2 allele with smoking behaviors in Japanese samples. Thus, researchers should try to eliminate or minimize the potential contribution of population stratification to the final results prior to analyzing the data. Further, evidence from an imaging study (Munro et al. 2006) indicated that dopamine release after stimulant exposure appears to be greater in males than in females. Several studies (Carpenter et al. 2006; Dluzen and Anderson 1997; Lerman et al. 1999) indicated that female subjects show greater estrogen-induced dopamine activation in the striatum. It is inferred that the higher estrogen concentration protects them from addiction when dopamine function is slack. We thus speculate that different sex ratios in various studies affected the final results. In addition to the aforementioned reasons, the relatively small populations in many reported association studies of the two genes with addictions might contribute to the inconsistent results.

Despite the progress in *DRD2/ANKK1* study in various addictions, many mysteries remain to be solved. We still have a long way to go to explore the nature of the links. First, more well-designed identification and replication studies, with large samples, of known and unknown variants across *DRD2/ANKK1* effects on addiction

need to be performed. Second, a large number of in vitro and in vivo studies should be completed to better understand how functional variants in *DRD2/ANKK1*, in particular *ANKK1*, affect brain dopaminergic system function at the molecular and cellular levels. Furthermore, it is important to notice that the association between addictions and genetic variants in *DRD2/ANKK1* is modulated by the environment and probably other genes as well; e.g., numerous studies (Finckh et al. 1996; Li 2000; Wong et al. 2000) showed environmental and genetic factors involved in the metabolism and pharmacodynamic effects of alcohol that could regulate the relation between AD and variants in *DRD2*. An improvement of our understanding of genetic and environmental factors underlying addiction would contribute to defining the most suitable approaches for prevention and novel medications for treating various addictive disorders.

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Chapter 9
Significant Contribution of Variants in Serotonin Transporter and Receptor Genes to Smoking Dependence

Abstract Although genetics contributes significantly to smoking addiction, the susceptibility genes and variants underlying it remain largely unknown. Many years of genome-wide and candidate gene-based association studies have implicated a number of genes and biological pathways in the etiology of nicotine and other addictions. In this chapter, we focus on current evidence, primarily from human genetic studies, supporting the involvement of variants in the serotonin transporter and receptor genes in the etiology of nicotine dependence (ND) based on both individual SNP- and haplotype-based association analysis, as well as gene-by-gene interaction studies. Current efforts aim not only to replicate these findings in independent samples but also to identify which variant(s) contributes to the etiology of ND and through what molecular mechanisms.

Keywords Serotonin · Serotonin transporter · Serotonin receptors · Epistasis · Gene interaction · Smoking dependence · Haplotype · SNPs · 5-hydroxytryptamine · *5-HT* · *5-HTTLPR* · *HTR3A* · *HTR3B*

1 Introduction

Drug addiction is a serious public health concern. According to the World Health Organization (2008), there were an estimated 2 billion alcohol abusers, 1.3 billion tobacco users, and 230 million illicit drug users worldwide in 2004. There is considerable evidence from family, twin, and adoption studies for the operation of genetic factors in the vulnerability to addiction and for the view that genetic factors contribute substantially to interindividual vulnerabilities, with an estimated moderate-to-high heritability for ND (see Chap. 3).

Many large twin studies have concluded that genetics contributes significantly to the risk of becoming a regular and dependent smoker. Meta-analysis of a dozen twin studies showed that both genetics and environment play important roles in smoking-related behaviors, with an estimated heritability for ND of 0.59 in male and 0.46 in female smokers, an average of 0.56 for the population as a whole (Li et al. 2003). In addition, ND is influenced by environmental factors, as well as by gene–gene and

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gene–environment interactions (Ho et al. 2010; Lessov-Schlaggar et al. 2008; Li et al. 2003; Sullivan and Kendler 1999; Swan et al. 2003).

2 Serotonin Transporter and Receptor Genes in Humans

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter that mediates rapid excitatory responses through specific receptors (i.e., 5-HT3 receptors). These receptors, unlike other serotonergic receptor classes, which are G-protein coupled (Barnes et al. 2009; Cravchik and Goldman 2000), belong to the superfamily of nicotinic acetylcholine (nACh), subtype A of the γ-aminobutyric acid ($GABA_A$) and glycine receptors. The serotonin-gated ion channel conducts primarily Na^+ and K^+, resulting in prompt neuronal depolarization followed by rapid desensitization and the release of stored neurotransmitter, suggesting an important role for this receptor system in the neuronal circuitry involved in drug abuse and addiction (Grant 1995).

The 5-HT3 receptors are colocalized with nAChRs on nerve terminals in several brain pathways involved in reward processing, including dopaminergic terminals in the striatum (Nayak et al. 2000). Although there is no evidence that these sites interact physically, cross-regulation may take place at a downstream molecular level (Dougherty and Nichols 2009; Nayak et al. 2000). Whereas 5-HT3 receptors assembled by 5-HT3_A subunits are uniformly located in various parts of the central and peripheral nervous systems, transcripts of the 5-HT3_A and 5-HT3_B subunits are co-expressed in the amygdala, caudate, and hippocampus, areas implicated in nicotine and other drug addictions, and form pharmacologically more potent heteropentameric receptors than the 5-HT3_A homomeric structures (Enoch et al. 2011). The genes encoding the 5-HT3_A and 5-HT3_B receptor subunits (namely, *HTR3A* and *HTR3B*) lie in a 90-kb region on chromosome 11q23.1 (Miyake et al. 1995).

Serotonin transporters, one major class of monoamine transporters, which regulate the availability of 5-HT in the synaptic cleft through reuptake, are encoded by the *SLC6A4* gene on chromosome 17q11.2 (Ramamoorthy et al. 1993). *SLC6A4* spans 37.8 kb and is composed of 14 exons (Lesch et al. 1994). The protein encoded by this gene, 5-HTT, is a transmembrane protein containing 630 amino acids. The expression of *SLC6A4* is regulated by at least three mechanisms: transcription regulatory elements in the promoter, differential splicing, and the use of different 3′ polyadenylation sites.

Of the two variable-number tandem repeat (VNTR) polymorphisms in the 5-HTT gene, one, in the transcriptional control region upstream of the coding region, called the 5-hydroxytryptamine transporter-linked polymorphic region (5-HTTLPR), is the most extensively investigated. The 5-HTTLPR variation is attributable to the presence (L-type allele) or absence (S-type allele) of a 44-bp insert. The S allele shows less transcriptional activity than the L allele, resulting in reduced function of the S/S compared with the L/L and L/S genotypes (Heils et al. 1996). The association between the 5-HTTLPR and numerous psychiatric disorders, including smoking behavior, has been investigated in a number of studies that yielded different

conclusions. Associations between the 5-HTTLPR L allele and smoking (Ishikawa et al. 1999) and between the L allele and coronary artery disease in smokers (Arinami et al. 1999) have been reported in a Japanese population. However, another study found no association between this polymorphism and cigarette smoking in Caucasians and African-Americans (AA) (Lerman et al. 1998). These conflicting data may be a consequence of various study populations and differences in phenotyping and grouping of genotypes. Two more recent studies have reported that this polymorphism modifies the effect of anxiety-related traits on smoking behavior (Hu et al. 2000; Lerman et al. 2000). Specifically, Lerman et al. (2000) reported that higher correlations exist between neuroticism and smoking motivations among smokers with the 5-HTTLPR S allele than smokers who are homozygous for the L allele. Meta-analysis of four studies (Hu et al. 2000; Ishikawa et al. 1999; Lerman et al. 1998) did not confirm the effect of this polymorphism (pooled odds ratio [OR] 1.15; 95% confidence interval [CI] 0.85, 1.56; P=0.35). More studies are required to determine whether there is an association between this polymorphism and smoking behavior.

3 Contribution of Variants in Serotonin Transporter and Receptor Genes to ND

To determine whether genetic variants in *SLC6A4*, *HTR3A*, and *HTR3B* contribute to the etiology of ND, Yang et al. (2013) genotyped 2 SNPs (5′-HTTLPR and rs1042173) in *SLC6A4*, 8 SNPs in *HTR3A*, and 7 SNPs in *HTR3B* in a sample consisting of 1366 AAs representing 402 nuclear families and 671 European Americans (EAs) representing 200 families and analyzed their association with ND, as assessed by smoking quantity (SQ), heaviness of smoking index (HSI), and Fagerström Test for Nicotine Dependence (FTND). Individual SNP-based association analysis revealed marginal associations of rs10160548 in *HTR3A* with SQ and HSI ($P = 0.030$ and 0.042, respectively) in the AA sample, rs11606194 in *HTR3B* with SQ and FTND ($P = 0.039$ and 0.028, respectively) in the pooled AA and EA samples, and 5-HTTLPR in *SLC6A4* with FTND ($P = 0.03$) in the EAs.

Following individual SNP-based association analysis, Yang et al. (2013) performed haplotype-based association analysis with the same genotyping data in the same samples, which revealed the following main findings. In AAs, there were two major haplotypes located in the 5′ region of *HTR3A* that were significantly associated with the three ND measures: (1) G-C-C-T-A-T, formed by SNPs rs1150226, rs1062613, rs33940208, rs1985242, rs2276302, and rs10160548 (LD block 3; Fig. 9.1), with a frequency of 19.5%, was associated significantly with SQ ($Z = 2.596$; $P = 0.009$), HSI ($Z = 3.027$; $P = 0.002$), and FTND ($Z = 2.824$; $P = 0.004$) in a dominant model; and (2) G-A, formed by SNPs rs1150220 and rs1176713 (LD block 4; Fig. 9.1), with a frequency of 66.6%, was significantly associated with SQ ($Z = 3.041$; $P = 0.002$,), HSI ($Z = 3.011$; $P = 0.003$), and FTND ($Z = 2.863$; $P = 0.004$). All these

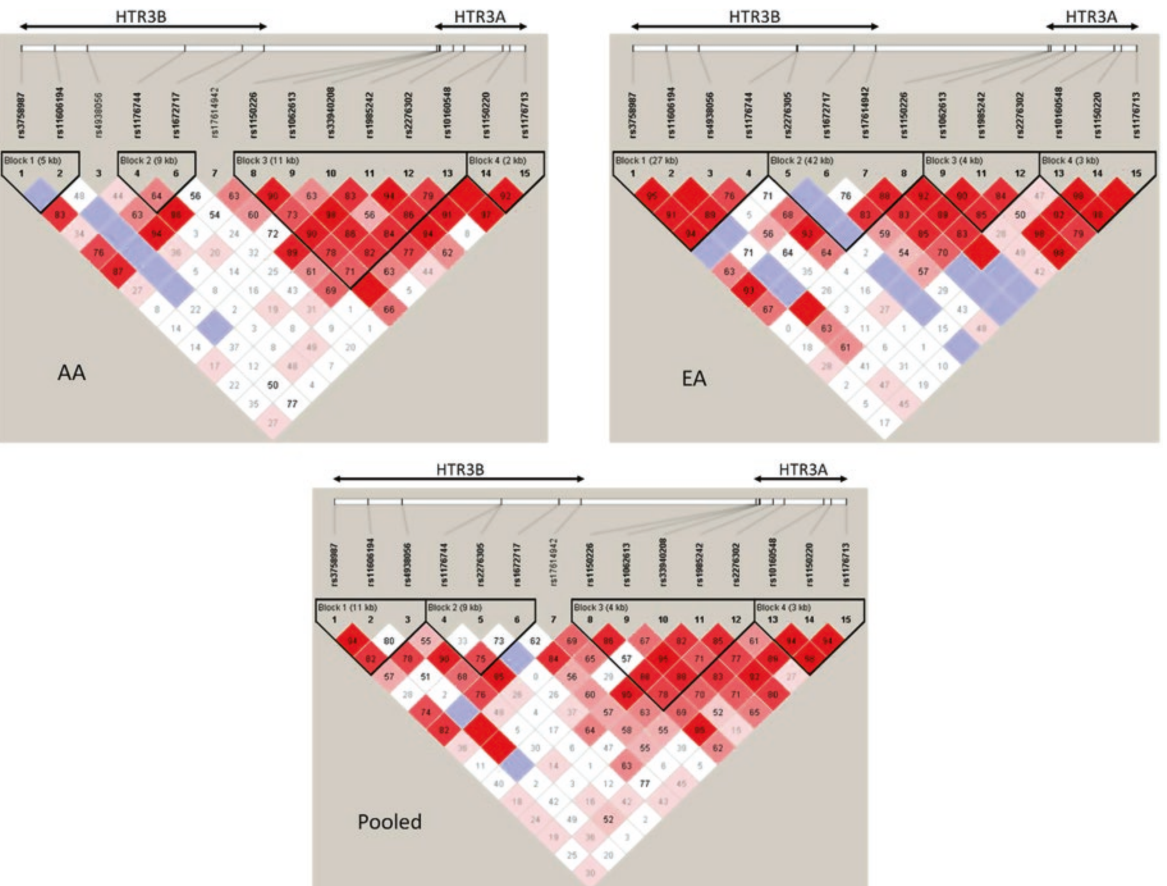

Fig. 9.1 Linkage disequilibrium structure for *HTR3B* and *HTR3A* SNPs in the AA, EA, and pooled samples of the Mid-South Tobacco Family study. Haploview was used to calculate all D′ values, and haplotype blocks were defined according to Gabriel et al. (2002). The number in each box represents the D′ value for each SNP pair surrounding that box (Reprinted from Yang et al. 2013)

haplotype-based associations remained significant after Bonferroni correction for multiple testing for each LD block. We also detected a nominally significant association of haplotype A-T-C-A-G-G in the LD block 3 of *HTR3A* with a frequency of 24.1% with SQ under the dominant model (Z = 1.996; P = 0.046).

For the EA sample, we found no haplotypes in *HTR3A* or *HTR3B* showing significant association with ND. In the pooled AA and EA sample, we found one haplotype, G-G-G, formed by SNPs rs10160548, rs1150220, and rs1176713 of *HTR3A*, with a frequency of 13.5%, significantly associated with SQ (Z = −2.377; *P* = 0.017), HSI (Z = −2.310; *P* = 0.021), and FTND (Z = −2.190; *P* = 0.029). However, none of them remained significant after Bonferroni correction.

4 Contribution of Variants in Serotonin Transporter and Receptor Genes for ND through Gene–Gene Interaction

Considering the biological and pharmacological functions of the three genes in regulating serotonin signaling, Yang et al. (2013) performed comprehensive interactive analysis among the 17 polymorphisms in *HTR3A*, *HTR3B*, and *SLC6A4* for their epistatic effect on the three ND measures in the same AA, EA, and pooled samples used for the studies described above. As shown in Table 9.1, the best interaction model detected for each sample shows a significant genetic interaction for all three ND measures, with an empirical $P < 0.01$, cross-validation consistency (CVC) of at least seven of ten, and test accuracies (TA) >50% based on 10^6 permutation tests, except for the model on FTND in the AA sample, where the empirical *P* value

Table 9.1 Detected best SNP combination of *SLC6A4*, *HTR3A*, and *HTR3B* associated with ND measures in EA, AA, and pooled sample on basis of test accuracy and empirical P value from 10^6 permutations

Sample	SNP combination	ND measure	Test accuracy	Cross-validation consistency (CVC)	Permutated P value
EA	*HTR3A*: rs1062613, rs1150220; *HTR3B*: rs1176744; *SLC6A4*: 5-HTTLPR, rs1042173	SQ	0.5678	7	0.003
		HSI	0.5699	9	0.002
		FTND	0.5703	10	0.002
AA	*HTR3A*: rs10160548; *SLC6A4*: 5-HTTLPR, rs1042173	SQ	0.5500	10	0.005
		HSI	0.5458	10	0.009
		FTND	0.5317	8	0.057
Pooled	*HTR3A*: rs1062613, rs10160548; *HTR3B*: rs1176744; *SLC6A4*: 5-HTTLPR, rs1042173	SQ	0.5516	8	0.00051
		HSI	0.5547	8	0.00025
		FTND	0.5479	10	0.00085

is 0.057. Of the three samples, the epistatic effect of the detected best interaction model for the three ND measures in the pooled sample appeared to be the strongest, with an empirical *P* value of 0.00025–0.00085.

In the pooled AA and EA samples, an interaction model consisting of five polymorphisms – rs1062613 and rs10160548 in *HTR3A*, rs1176744 in *HTR3B*, and 5-HTTLPR, and rs1042173 in *SLC6A4* – showed significant epistatic effects on all three ND measures. Interestingly, the minor allele frequencies of these five polymorphisms are quite high, with the lowest frequency being 0.211 for rs1042173 in *SLC6A4* in the AA sample and 0.244 for rs1062613 in *HTR3A* in the EA sample. Of them, three polymorphisms have been demonstrated to alter the expression of the RNA or protein encoded by the respective genes (Niesler et al. 2001). For example, the rs1062613 is a translation regulatory variant located in an open reading frame upstream of the translation initiation site of *HTR3A* mRNA (Niesler et al. 2001). The two polymorphisms in *SLC6A4* alter 5-HTT expression through transcription regulation for 5-HTTLPR and degradation of mRNA transcripts for rs1042173 (Heils et al. 1996, 1997; Seneviratne et al. 2009; Vallender et al. 2008). Of the remaining SNPs, rs10160548 is located in intron 6 near an intron–exon boundary. It is thus reasonable to speculate that it alters the expression of functional *HTR3A* transcripts through alternative splicing. The rs1176744 in *HTR3B* does not alter expression, but it substantially changes serotonergic signaling through altered gating kinetics of the 5-HT_{3AB} receptor complex (Krzywkowski et al. 2008).

By analyzing the AA and EA samples independently, Yang et al. (2013) revealed slightly different interaction models for each ethnic sample. In the AA sample, there was a significant interactive effect of polymorphisms rs10160548 in *HTR3A* and 5-HTTLPR and rs1042173 in *SLC6A4* on all three ND measures. Although the two *SLC6A4* polymorphisms were also included in the best interaction model detected in the EA sample, the model contained three additional loci: rs1062613 and rs1150220 in *HTR3A* and rs1176744 in *HTR3B*. In previously reported studies, SNP rs1062613 in *HTR3A* was associated with several psychiatric disorders in individuals of European descent (Gatt et al. 2010; Walstab et al. 2010). Yet whether rs1062613 has ethnicity-specific *cis*-acting effects on the differential extents of translation of *HTR3A* in AAs and EAs remains to be characterized. However, inclusion of rs1062613 in the best interaction model in the pooled samples, with even stronger interaction effects than were seen in EAs only, argues against this possibility. The other *HTR3A* SNP, detected only in the EA sample, was rs1150220, which is moderately correlated with rs10160548 in both EAs and AAs ($r^2 = 0.42$ in AAs and 0.51 in EAs) in an LD block located at the 3′ end of the *HTR3A* gene. The second main difference between the AA and EA samples was the absence of *HTR3B* rs1176744 in the best model for AAs. Although SNPs rs1176744 and 5-HTTLPR in *SLC6A4* have been significantly associated with alcohol dependence in AAs (Enoch et al. 2011), no significant association of these two polymorphisms with ND was revealed in the AA, EA, or pooled samples, except for 5-HTTLPR, which showed a marginal association with FTND in the EA sample. However, these genetic interaction analyses demonstrated that the two polymorphisms in *SLC6A4* play an important role in ND through interactions with other SNPs in *HTR3A* and *HTR3B* in the AA, EA, and pooled samples.

5 Comparison of Main vs. Interactive Effect of Variants in Transporter and Receptors on ND

The most important finding from the study reported by Yang et al. (2013) is that none of the polymorphisms included in the epistatic models shown in Table 9.1 was significant at the individual locus level. Significant epistatic effects of variants without major genetic effect have become an increasingly identified phenomenon in studies of complex disorders (Li et al. 2008; Steen 2012; Zuk et al. 2012). For example, several studies have shown marginal or no association of the polymorphism 5-HTTLPR in *SLC6A4* with ND (Gerra et al. 2005; Trummer et al. 2006; Yang et al. 2013). Yet, as clearly demonstrated by the gene-by-gene interaction analysis, the effect of 5-HTTLPR on ND is highly significant when its epistatic effect is taken into consideration. Another unique strength of these findings is that the interaction models detected in AAs, EAs, and the pooled sample were highly significant across multiple ND measures, providing further support for their role in smoking-related behaviors.

The biological basis for the genetic interaction effect we detected can be explained by the actions of nicotine on serotonergic signaling. Nicotine competes with its natural ligand serotonin for 5-HT$_3$ receptors (Breitinger et al. 2001). Depending on whether the 5-HT$_3$ receptors are located pre- or post-synaptically, nicotine binding can result in either the release of various neurotransmitters or changes in the propagation of fast-acting serotonergic signals along the postsynaptic neuron. The availability of synaptic serotonin for binding to the 5-HT$_3$ receptors is modulated by the presynaptic 5-HTTs. Prior studies have found mixed effects of chronic nicotine exposure on the density of 5-HTTs, thus regulating the amount of synaptic serotonin available for action on the 5-HT$_3$ receptors. For example, Semba and Wakuta (2008) reported a reduction in the density of 5-HTTs in the rat brain, whereas two other studies reported an elevation in 5-HTTs (Awtry and Werling 2003; Slotkin and Seidler 2010). On the other hand, Staley et al. (2001) reported an elevation of 5-HTTs in the human brain; whereas in human platelets, they are reduced (Patkar et al. 2003). Serotonin plays a crucial role in mediating cognitive behavioral functions, stress response, mood, appetite, and motor functions (Jasinska et al. 2012). Thus, the interactions among the three genes may represent the interacting biological effects of nicotine on fast-acting serotonergic signaling in ND.

6 Concluding Remarks

In summary, by examining the association of variants in *HTR3A*, *HTR3B*, and *SLC6A4* with ND at both the individual SNP and haplotype levels, only marginal association of variants in the three genes with the two addictive phenotypes was revealed in one of the studied samples. However, when these variants were examined interactively through the gene–gene interaction approach, a combination of

functional polymorphisms in the three genes with significant interaction effects on ND was identified. This strongly indicates that these genetic variants play a significant role in ND through an epistatic effect. It is important to investigate genetic epistatic effects when one searches for susceptibility loci for a complex trait such as ND, as described in this chapter. Importantly, SNPs rs10160548 in *HTR3A*, rs1176744 in *HTR3B*, and 5-HTTLPR and rs1042173 in *SLC6A4* were found to be significant, influencing ND through epistasis.

In spite of this progress in molecular genetic studies of addictions, we still have a long way to go, and there are many challenges that remain to be surmounted (Ho et al. 2010; Li 2010; van der Zwaluw and Engels 2009). These challenges include (1) further identification and replication of known and unknown genes in the serotonin receptor and other signaling pathways and functional variants (including rare variants) for various addictive disorders through high-throughput approaches such as association study and deep sequencing analysis, (2) study of copy number variations and their impact on gene expression in serotonin signaling pathway and other addiction-related signaling pathways, (3) better understanding of the mechanisms underlying addictions at the molecular and cellular levels using both in vitro and in vivo approaches, and (4) determining appropriate ways of defining environmental factors such that we can assess how gene–environment interaction affects addiction. An improvement of our understanding of the genetic and environmental factors underlying drug addiction has considerable potential to reduce morbidity and death greatly by providing the most suitable methods for prevention and novel medications for treating different addictive disorders.

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Chapter 10
Converging Findings from Linkage and Association Analyses on Susceptibility Genes for Smoking Addiction

Abstract To search for susceptibility genes and loci for nicotine addiction, many genetic approaches have been used, which include genome-wide linkage, candidate gene association, GWAS, and targeted sequencing. Through these approaches, many genes and chromosomal regions have been revealed. In this chapter, we first summarize the literature on genetic studies for all smoking-related phenotypes using different approaches by highlighting the converging results obtained by different approaches and then offer new hypotheses that have emerged across the allelic spectrum, including common and rare variants. It is our hope that the insights we obtained by putting together results from diverse approaches can be applied to other complex diseases/traits. In sum, developing a genetic susceptibility map and keeping it updated are an effective way to keep track of what we know about the genetics of smoking addiction and what the next steps might be with new approaches.

Keywords Linkage analysis · GWAS · Genetic association · Next-generational sequencing · Target sequencing · Meta-analysis · Functional SNPs · Candidate genes · Nicotine dependence · Missing heritability · Gene–gene interaction · Gene–environmental interaction

1 Introduction

Since the 1980s, a broad scientific consensus has been established that ND is the primary factor maintaining smoking behavior. We and others have shown strong evidence for the involvement of genetics in ND, with an average heritability of 0.56 (Carmelli et al. 1992; Li et al. 2003). In the past dozen years, considerable efforts have been exerted to identify the genetic factors underlying ND. However, only three widely accepted "successes," i.e., the neuronal nicotinic acetylcholine receptor gene clusters on chromosomes 15 (*CHRNA5/A3/B4*) (Berrettine et al. 2008; Bierut et al. 2008; Chen et al. 2012c; David et al. 2012; Keskitalo et al. 2009; Li et al. 2010a, b; Liu et al. 2010; Saccone et al. 2007, 2010; TAG 2010; Thorgeirsson et al. 2008, 2010; Weiss et al. 2008) and 8 (*CHRNB3/A6*) (Cui et al. 2013; Culverhouse et al. 2014; Hoft et al. 2009; Rice et al. 2012; Saccone et al. 2007, 2010; Thorgeirsson et al. 2010; Zeiger et al. 2008) and the genes encoding nicotine-metabolizing enzymes on chromosome 19 (*CYP2A6/A7*) (Bloom et al. 2014; Chen

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et al. 2014; Kumasaka et al. 2012; TAG 2010; Thorgeirsson et al. 2010), meet the community standards for significance and replicability (Chanock et al. 2007). These few triumphs stand in contrast to the limited heritability they explain; e.g., the most significant synonymous SNP rs1051730 ($P = 2.75 \times 10^{-73}$) in *CHRNA3* accounted for only 0.5% of the variance in cigarettes smoked per day (CPD) in a meta-analysis of 73,853 subjects (TAG 2010). Researchers have suggested that "missing heritability" is merely hidden and that additional loci can be discovered using GWAS with larger samples (Lee et al. 2012; Yang et al. 2010), not to mention that the largest ND GWAS to date included 143,023 subjects (TAG 2010), and many relevant genetic loci have been revealed with other experimental approaches, such as genome-wide linkage, hypothesis-driven candidate gene association, and targeted sequencing. Although many non-GWAS findings have an uncertain yield or failed to be replicated, sorting out genetic loci with evidence from multiple approaches is not only essential but also more cost effective than pursuing a formidable sample size for GWAS.

2 Genome-Wide Linkage Studies on Smoking Addiction

For many years, linkage analysis was the primary approach for the genetic mapping of both Mendelian and complex traits with familial aggregation (Gelernter 2015; Ott et al. 2015). This method was largely supplanted by the wide adoption of GWAS in the middle 2000s. In 2008, we published a comprehensive review of more than 20 published genome-wide linkage studies of smoking behavior and identified 13 regions, located on chromosomes 3–7, 9–11, 17, 20, and 22, suggestively or significantly linked with various ND measurements in at least two independent samples (Li 2008). Since then, only one genome-wide linkage study has been reported, by Hardin et al. (2009), finding a linked spot in the same region as in their previous analysis (6q26) using the same sample but a different phenotype (Swan et al. 2006). In addition, Han et al. (2010) conducted a meta-analysis of 15 genome-wide linkage scans of smoking behavior and identified two suggestive (5q33.1–5q35.2 and 17q24.3–q25.3) and one significant (20q13.12–q13.32) linkage regions. In fact, the regions on chromosomes 5 and 20 expand two of the regions reported in our 2008 review. The region on chromosome 17 reported by Han et al. (2010) verified one of the regions detected in only one sample before 2008, which makes it a newly nominated linkage peak (Table 10.1) (Li 2008). Figure 10.1 shows updated linkage results for ND assessed by various ND measures.

Table 10.1 Information on the nominated linkage regions updated according to Li (2008)

Chromosome	Marker or marker region	Chr. bands	Phenotype
3	D3S1763–D3S1262	3q26–q27	DSM-IV ND, SQ
4	D4S403–D4S2632, D4S244	4p15–q13.1	FTND, CPD
5 (region 1)	D5S1969, D5S647, D5S428	5q11.2–q14	SQ, smoking status, FTND
5 (region 2)[a]	D5S400, D5S1354	5q33.1–q35[a]	FTND, CPD
6	D6S1009, D6S1581–D6S281, D6S446	6q23.3–q27	Smoking status, FTND, withdrawal severity
7	D7S486, D7S636	7q31.2–q36.1	FTND, DSM-IV
9 (region 1)	D9S2169–D9S925, D9S925–D9S319	9p21–p24.1	FTND, HSI, SQ
9 (region 2)	D9S257–D9S910, D9S283, D9S64, D9S1825	9q21.33–q33	SQ, FTND, smoking status
10	D10S1432, D10S2469/CYP17, D10S597, D10S1652-D10S1693, D10S129-D10S217	10q21.2–q26.2	SQ, FTND, smoking status
11	D11S4046, D11S4181, D11S2362-D11S1981, D11S1999-D11S1981, D11S2368–D11S2371, D11S1392–D11S1344, D11S1985–D11S2371	11p15–q13.4	FTND, SQ
17 (region 1)	GATA193, D17S974–D17S2196, D17S799–D17S2196, D17S799–D17S1290	17p13.1–q22	CPD, SQ, HSI
17 (region 2)[a]	D17S968	17q24.3–q25.3[a]	Smoking status
20[a]	D20S119–D20S178, D20S481–D20S480	20q13.12–q13.32[a]	CPD, SQ
22	D22S345–D22S315, D22S315–D22S1144	22q11.23–12.1	CPD, age at first cigarette

This table was modified from Table 3 of Li (2008)

[a]Denotes linkage regions expanded or newly ascertained after evaluating results published after our 2008 review. Genomic positions for microsatellite markers and corresponding chromosome bands were obtained through the UCSC Genome Browser (http://genome.ucsc.edu/), which are in the GRCh37/hg19 assembly

Chr chromosome, *CPD* cigarettes smoked per day, *DSM* Diagnostic and Statistical Manual (American Psychiatric Association), *FTND* Fagerström Test for Nicotine Dependence, *HSI* Heaviness of Smoking Index, *SQ* smoking quantity

Fig. 10.1 The ND genetic susceptibility map with nominated linkage peaks and candidate genes, as suggested by genome-wide linkage, hypothesis-driven candidate gene association (CAS), genome-wide association study (GWAS), and targeted sequencing (next-generation sequencing [NGS]) studies. Linkage peaks are marked in light gray; CAS, GWAS, and NGS results are presented as gene names at the outer, middle, and inner rings, respectively

3 Candidate Gene-Based Association Studies on Smoking Addiction

Candidate gene association studies usually have moderate sample sizes and are much cheaper than GWAS, where the genes examined are selected according to the linkage/GWAS study results or biological hypotheses. However, because of population heterogeneity and liberal statistical thresholds (compared with GWAS) that often are applied, hypothesis-driven candidate gene association studies generally are considered to have an uncertain yield (Sullivan et al. 2012). On the other hand, the abundant results obtained using this approach provide greater depth of exploration of potential targets and offer valuable replication for other unbiased approaches, e.g., genome-wide linkage study and GWAS.

To eliminate concerns about potential false-positive results, especially for studies reported in earlier years, we focused primarily on the genes showing significance in at least two independent studies with a sample size of ≥1000 or within (or close to) nominated linkage regions or overlapping with GWAS results but with a sample size of ≥500 based on the statistical thresholds set by each study. Because the reported sex-averaged recombination rate is 1.30 ± 0.80 cM/Mbp (Yu et al. 2001), in this report, we defined candidate genes within 2 Mbp of any linkage region as "within" and 2–5 Mbp as "close to." The sample size requirement was determined with the following parameters: two-tailed $\alpha = 0.05$, population risk = 0.30, minor allele frequencies (MAFs) = 0.20, and genotypic relative risk = 1.3 with an approximate odds ratio (OR) of 1.5 or 0.7, which is similar to the statistics usually found in candidate gene association studies. For a statistical power of 0.80 ($\beta = 0.20$) using the allelic test, the minimum sample size for a case-control study is 1062, with equal numbers of cases and controls. Of the reported 201 candidate gene association studies, only 88 have had a sample size of 1000 or more. Considering the detected power of 0.54 for a sample size of 500 under the dominant genetic model, we also included genes implicated in studies with 500–1000 subjects if the genes were located in a nominated linkage peak (Li 2008) or overlapped with GWAS signals. In total, 34 genetic loci with 43 genes met the criteria (Table 10.2 and Fig. 10.1), which were assigned to the following four groups.

3.1 Neurotransmitter System Genes

Dopaminergic System: The dopaminergic system has long been acknowledged to play a critical role in nicotine addiction (Dani 2003). The most studied gene in this system is *DRD2*, located on chromosome 11q23.2 within a modest linkage peak (Gelernter et al. 2007). The intriguing polymorphism *Taq*1A is located in *ANKK1* near *DRD2*, leading to an amino acid change in *ANKK1* (Neville et al. 2004). Several other variants and haplotypes in regions adjacent to *DRD2*, within *TTC12* and *ANKK1*, or downstream of *DRD2* have been associated with smoking-related

Table 10.2 Significant candidate gene association results for ND-related phenotypes

Gene	Chr.	Variants
Neurotransmitter system genes		
Dopaminergic system		
TTC12	11q23.2	rs4245150 (intergenic); rs17602038 (intergenic); rs2303380; rs10502172 (intronic)
DRD2	11q23.2	rs1800497 (*Taq*1A) (near 5′-gene); rs4938012 (near 5′-gene)
ANKK1	11q23.2	rs2734849 (missense); rs4938015; rs11604671; rs1800497 (Taq1A) `(missense); rs1799732 (-141C Ins/Del) (missense)
DRD1	5q35.2	rs686 (3’-UTR)
DRD4	11p15.5	VNTR (exon 3)
DBH	9q34.2	rs1541333 (intronic); rs3025382 (intronic); rs4531 (missense); rs5320 (missense)
DDC	7p12.1	rs12718541 (intronic); rs921451 (intronic)
COMT	22q11.21	rs737865-rs165599; rs4680 (missense)
PPP1R1B	17q12	rs2271309-rs907094-rs3764352-rs3817160
OPRM1	6q25.2	rs1799971 (missense); rs510769 (intronic)
GABAergic system		
GABBR2	9q22.33	rs1435252 (intronic); rs3750344 (synonymous)
GABARAP	17p13.1	rs222843 (near Gene-5)
GABRA4	4p12	rs3762611 (near Gene-5)
Serotonergic system		
HTR3A	11q23.2	rs1150226-rs1062613-rs33940208-rs1985242-rs2276302-rs10160548
HTR5A	7q36.2	rs6320 (synonymous)
SLC6A4	17q11.2	5-HTTLPR+intronic VNTR; 5-HTTLPR
Glutamatergic system and other		
GRIN3A	9q31.1	rs17189632 (intronic)
GRIN2B	12p13.1	rs17760877 (intronic)
NRXN1	2p16.3	rs6721498 (intronic); rs2193225 (intronic)
Nicotinic receptor (nAChR) subunit and other cholinergic system genes		
CHRNA3	15q25.1	rs1051730 (synonymous); rs578776 (3′-UTR); rs3743078 (intronic); rs11637630 (intronic)
CHRNB4	15q25.1	rs1948 (3′-UTR); rs17487223 (intronic)
CHRNA5	15q25.1	rs16969968 (missense); rs16969968-rs680244; rs951266 (intronic); rs569207
CHRNB3	8p11.21	rs4950 (5′-UTR); rs7004381 (near Gene-5); rs13277254 (near 5′-gene); rs6474412 (near Gene-5); rs13280604 (intronic); rs13273442 (near 5′-gene); rs4736835 (near 5′-gene)
CHRNA6	8p11.21	rs892413 (intronic)
CHRNA4	20q13.33	rs1044397 (synonymous); rs2236196 (3′-UTR); rs1044396 (missense)
CHRNB1	17p13.1	rs17732878 (near 3′-gene); rs2302763 (intronic)
CHRM1	11q12.3	rs2507821-rs4963323-rs544978-rs542269-rs2075748-rs1938677
CHRM2	7q33	rs1378650 (near 3′-gene)

(continued)

Table 10.2 (continued)

Gene	Chr.	Variants
Nicotine metabolism genes		
EGLN2-CYP2A6-CYP2B6	19q13.2	rs1801272 (missense); rs28399433 (near Gene-5); genotype-based metabolism; CYP2A6*12 (crossover with CYP2A7); CYP2A6*1B (conversion)
CYP2B6	19q13.2	rs4802100 (near 5′-gene)
EGLN2	19q13.2	rs3733829 (intronic)
MAPK signaling pathway and other genes		
BDNF	11p14.1	rs6265 (missense); rs6484320-rs988748-rs2030324-rs7934165
NTRK2	9q21.33	rs1187272 (intronic)
ARRB1	11q13.4	rs528833-rs1320709-rs480174-rs5786130-rs611908-rs472112
MAP3K4	6q26	rs1488 (3′-UTR)
SHC3	9q22.1	rs1547696 (intronic)
DNM1	9q34.11	rs3003609 (synonymous)
TAS2R38	7q34	Haplotype conferring intermediate taste sensitivity (AAV); taster (PAV) and non-taster (AVI) haplotypes
APBB1	11p15.4	rs4758416 (intronic)
PTEN	10q23.1	rs1234213 (intronic)
NRG3	10q23.1	rs1896506 (intronic)

phenotypes (David et al. 2010; Ducci et al. 2011; Gelernter et al. 2006; Huang et al. 2009; Saccone et al. 2007). Besides *DRD2*, a modest number of studies have shown significant associations between ND measures and other dopamine receptor genes, such as *DRD1* (Huang et al. 2008a) and *DRD4* (Das et al. 2011; David et al. 2008b; Ellis et al. 2011), and genes involved in dopamine metabolism, including dopamine β-hydroxylase (*DBH*) (Ella et al. 2012; Leventhal et al. 2014; Saccone et al. 2007), DOPA decarboxylase (*DDC*) (Ma et al. 2005; Yu et al. 2006) and catechol-O-methyl transferase (*COMT*) (Amstadter et al. 2009; Berrettini et al. 2007; Beuten et al. 2006b; Munafo et al. 2011a; Nedic et al. 2010; Omidvar et al. 2009). All of these genes are within or close to the nominated linkage peaks (Li 2008) except for *DBH* and *DDC*, which have received support from GWAS results (TAG 2010) and as ND-associated genes from two independent studies with sample sizes ≥1000 (Ellis et al. 2011; Leventhal et al. 2014; Saccone et al. 2007).

Huang et al. (2008b) implicated *DRD3* as a susceptibility gene for ND, but this result has not yet been replicated. Meanwhile, Stapleton et al. (2007) showed a significant association of a dopamine transporter gene (*SLC6A3*) with smoking cessation in a meta-analysis of 2155 subjects (80% of European ancestry), although this finding received only weak support from another study on age at smoking initiation in 668 Asians (Ling et al. 2004). This gene group includes two others, protein phosphatase 1 regulatory subunit 1B (*PPP1R1B*) and μ-opioid receptor (*OPRM1*), on the basis of their functional connections with dopamine in studies of other addictive substances. *PPP1R1B*, also known as dopamine- and cAMP-regulated neuronal phosphatase (*DARPP-32*), encodes a key phosphoprotein involved in the regulation of several signaling cascades for dopaminoceptive neurons in several areas of the

brain, which also is required for the biochemical effects of cocaine (Farris et al. 2015). Activation of OPRM1 in the ventral tegmental area (VTA) suppresses the activity of inhibitory GABAergic interneurons, resulting in disinhibition of dopamine neurons and dopamine release from terminals in the ventral striatum (Ramchandani et al. 2011). *OPRM1* A118G variation is a genetic determinant of the striatal dopamine response to alcohol in men (Ramchandani et al. 2011), with a preliminary study of tobacco smoking confirming this result (Domino et al. 2012). Although we believe in the importance of the abovementioned genes in ND based on rigorous scientific evidence, the inconsistent results are worth further examination (Breitling et al. 2009a; Huang et al. 2005; Marteau et al. 2012; Munafo et al. 2013; Ton et al. 2007).

GABAergic and Serotonergic Systems: For the GABAergic system, variants in the $GABA_B$ receptor subunit 2 (*GABBR2*) (Beuten et al. 2005a), $GABA_A$ receptor-associated protein (*GABARAP*) (Lou et al. 2007), and $GABA_A$ receptor subunits alpha-2 (*GABRA2*) and alpha-4 (*GABRA4*) (Agrawal et al. 2008, 2009; Saccone et al. 2007) are significantly associated with different ND phenotypes. Chapter 7 summarizes the significance of the GABAergic system in ND and alcohol dependence. The serotonergic system is implicated in susceptibility to ND because nicotine increases serotonin release in the brain, and symptoms of nicotine withdrawal are associated with diminished serotonergic neurotransmission (Iordanidou et al. 2010). Genes encoding serotonin receptor 3A, ionotropic (*HTR3A*) (Yang et al. 2013), 5A, G-protein-coupled (*HTR5A*) (Saccone et al. 2007), and serotonin transporter (*SLC6A4*) (Bidwell et al. 2012; Daw et al. 2014; Kremer et al. 2005) showed significant association with smoking-related behaviors. All of these seven genes of the GABAergic and serotonergic systems are within or close to the nominated linkage peaks (Li 2008), which strengthen the validity of the associations, although two studies reported negative results (David et al. 2008a; Trummer et al. 2006). Another gene worth mentioning from this group is serotonin receptor 2A, G-protein-coupled (*HTR2A*), which is within a modest linkage peak (13q14) suggested by Li et al. (2006) and was significantly associated with smoking status in a Brazilian sample of 625 subjects (do Prado-Lima et al. 2004). Replication in larger samples is needed to confirm the association of this gene with ND.

Glutamatergic System and Related Genes: Two glutamate receptors, ionotropic, NMDA 3A (*GRIN3A*), within the nominated linkage peak on 9q21.33–q33 (Li 2008), and NMDA 2B (*GRIN2B*), suggested by one GWAS (Vink et al. 2009) and close to a modest linkage peak on 12p13.31–13.32 (Li et al. 2008b), are significantly associated with scores on the FTND (Grucza et al. 2010; Ma et al. 2010). More genes in the glutamatergic system, such as *GRIN2A*, *GRIK2*, *GRM8*, and *SLC1A2*, show suggestive association with smoking behavior in the GWAS reported by Vink et al. (2009) but without significant replication in candidate gene association studies. Accumulating evidence suggests that blockade of glutamatergic transmission attenuates the positive reinforcing and incentive motivational aspects of nicotine, inhibits the reward-enhancing and conditioned rewarding effects of the drug, and blocks nicotine-seeking behavior (Li et al. 2014). More attention may be paid to this neurotransmitter system in the future.

In the catch-all part, after showing suggestive association in the first ND GWAS (Bierut et al. 2007), neurexin 1 (*NRXN1*) association has been replicated in two independent studies with more than 2000 subjects of three ancestries: African, Asian, and European (Nussbaum et al. 2008; Sato et al. 2010). Although neurexin 3 (*NRXN3*) also showed a significant association with the risk of being a smoker (Docampo et al. 2012), this finding has not been verified in any other ND samples, and *NRXN3* is not within any detected linkage peak (Li 2008). Neurexins are cell-adhesion molecules that play a key role in synapse formation and maintenance and have been implicated in polysubstance addiction (Liu et al. 2005).

3.2 Nicotinic Receptor (nAChR) Subunit and Other Cholinergic System Genes

As nAChR subunit gene clusters on chromosomes 15 (*CHRNA5/A3/B4*) and 8 (*CHRNB3/A6*) are major discoveries from ND GWAS, their candidate association results will be discussed together with the GWAS results. Significant associations of variants in two other subunit genes (*CHRNA4* and *CHRNB1*) did not approach genome-wide significance ($P < 5 \times 10^{-8}$), but they are both close to nominated linkage peaks (Li 2008). Association of *CHRNA4* with ND, close to the nominated linkage peak on 20q13.12–13.32 (Li 2008), has been demonstrated in five independent studies (Table 10.2) (Breitling et al. 2009b; Feng et al. 2004; Grucza et al. 2010; Kamens et al. 2013; Li et al. 2005). Variants within *CHRNB1*, located close to the nominated linkage peak on 17p13.1–q22 (Li 2008), are significantly associated with FTND and CPD scores (Grucza et al. 2010; Lou et al. 2006). Two other genes encoding nAChR subunits, *CHRNB2* and *CHRNA2*, although associated with ND-related phenotypes in two studies (Ehringer et al. 2007; Wang et al. 2014), are not within any detected linkage peaks and have no replication studies reported that are of the required sample size. Thus, these two genes are considered to have only weak evidence of involvement in ND and therefore are not included in Fig. 10.1 or Table 10.2. Besides nAChR subunit genes, two cholinergic receptors, muscarinic 1 (*CHRM1*) and 2 (*CHRM2*), were found to be significantly associated with CPD and FTND, respectively (Grucza et al. 2010; Lou et al. 2006). They are within nominated linkage peaks as well (Li 2008). However, because of the inadequacy of knowledge of their biological functions, they have been less investigated.

3.3 Nicotine Metabolism Genes

Of the nicotine metabolism genes, those encoding nicotine-metabolizing enzymes (*CYP2A6* and *CYP2B6*) have been the most investigated (Ray et al. 2009). Six studies have provided consistent evidence that variants leading to reduced or absent

CYP2A6 activity are associated with various smoking-related phenotypes, including the nicotine metabolite ratio (Johnstone et al. 2006), time to smoking relapse (Chen et al. 2014), exhaled carbon monoxide (CO) (Bloom et al. 2014), initial subjective response to nicotine (Bidwell et al. 2012), FTND (Saccone et al. 2007), and CPD (Chen et al. 2012a). All six samples consisted of subjects of European descent (Table 10.1). The negative result of *CYP2A6* in the 2004 meta-analytic review contrasts with the findings from more recent studies, which we believe offer stronger statistical evidence (Carter et al. 2004). Such significant association of variants in the *EGLN2–CYP2A6–CYP2B6* region with ND is corroborated by GWAS results, as discussed in the next section (Kumasaka et al. 2012; Thorgeirsson et al. 2010).

3.4 Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway and Other Genes

Further, we want to acknowledge studies implicating other genes in ND, including brain-derived neurotrophic factor (*BDNF*) (Beuten et al. 2005b; Zhang et al. 2012), neurotrophic tyrosine kinase, receptor type 2 (*NTRK2*) (Beuten et al. 2006a), arrestin β1 (*ARRB1*) (Sun et al. 2008), *MAP3K4* (Grucza et al. 2010), *SHC3* (Li et al. 2007), dynamin 1 (*DNM1*) (Xu et al. 2009), taste receptor type 2, member 38 (*TAS2R38*) (Mangold et al. 2008), amyloid β-precursor protein-binding, family B, member 1 (*APBB1*) (Chen et al. 2008), *PTEN* (Zhang et al. 2006), and neuregulin 3 (*NRG3*) (Turner et al. 2014). It is worth noting that the first five of these genes belong to the MAPK signaling pathway, which is significantly enriched in involvement with four drugs subject to abuse, namely, cocaine, alcohol, opioids, and nicotine (Li et al. 2008a).

4 Genome-Wide Association Studies (GWAS)

Since the first GWAS was published in 2005 (Klein et al. 2005), this technique, using millions of SNPs, became the preferred mapping tool for complex disease/traits (Ott et al. 2015). As of October 2015, nine published GWASs and meta-GWASs have yielded 11 genetic loci carrying variants of genome-wide significance (GWS; $P < 5 \times 10^{-8}$) associated with relevant ND phenotypes in subjects of European, African, and East Asian ancestries (Table 10.3 and Fig. 10.1). However, only three loci were replicated in more than two independent GWASs or meta-GWASs, among which the *CHRNA5/A3/B4* cluster has the most evidence of significance.

Before the GWAS reports, Saccone et al. (2007) reported significant association of a 3′-UTR variant (rs578776) in *CHRNA3* with dichotomized FTND in smokers in a candidate gene association study examining 348 genes. Then, in the GWAS era,

Table 10.3 Significant genome-wide association study (GWAS) findings for ND-related phenotypes

Population	Phenotype	Nearest gene	Chr.	SNP [effect allele]	Variant type	*P* value	Effect size
European	CPD	*CHRNA5/A3/B4*	15q25.1	rs1051730[A]	Synonymous	2.8×10^{-73}	β = 1.02
				rs16969968[G]	Missense	5.6×10^{-72}	β = 1.00
				rs6495308[T]	Intronic	5.8×10^{-44}	β = 0.73
				rs55853698	5′-UTR	1.3×10^{-16}	
		CYP2A6, *EGLN2*, *RAB4B*	19q13.2	rs4105144[C]	Intergenic	2.2×10^{-12}	β = 0.39
				rs7937[T]	3′-UTR	2.4×10^{-9}	β = 0.24
				rs3733829[G]	Intronic	1.0×10^{-8}	β = 0.33
		LOC100188947	10q23.32	rs1329650[G]	Intronic	5.7×10^{-10}	β = 0.37
				rs1028936[A]	Intronic	1.3×10^{-9}	β = 0.45
		PDE1C	7p14.3	rs215605[G]	Intronic	5.4×10^{-9}	β = 0.26
		CHRNB3/A6	8p11.21	rs13280604[A]	Intronic	1.3×10^{-8}	β = 0.31
				rs6474412[T]	Intergenic	1.4×10^{-8}	β = 0.29
	FTND	*CACNA2D1*	7q21.11	rs13225753	Intergenic	3.5×10^{-8}	NA
	Smoking initiation	*BDNF*	11p14.1	rs6265[C]	Missense	1.8×10^{-8}	OR = 1.06
	Smoking cessation	*DBH*	9q34.2	rs3025343[G]	Intergenic	3.6×10^{-8}	OR = 1.12
	NMR	*CYP2A6*, *CYP2B6*, *CYP2A7*, *EGLN2*, *NUMBL*	19q13.2	rs56113850[C]	Intronic	5.8×10^{-86}	β = −0.65
African-American	CPD	*CHRNA5/A3/B4*	15q25.1	rs2036527[A]	Intergenic	1.8×10^{-8}	β < 1.00
	FTND	*C14orf28*	14q21.2	rs117018253	Intergenic	4.7×10^{-10}	NA
		CSGALNACT1, *INTS10*	8p21.3	rs6996964	Intergenic	1.1×10^{-9}	NA
		DLC1	8p22	rs289519	Intronic	4.5×10^{-8}	NA
European and African-American	Dichotomized FTND	*CHRNB3*	8p11.21	rs1451240[A]	Intergenic	6.7×10^{-16}	OR = 0.65

(continued)

Table 10.2 (continued)

Population	Phenotype	Nearest gene	Chr.	SNP [effect allele]	Variant type	*P* value	Effect size
Japanese	CPD	*CYP2A6*, *CYP2A7*	19q13.2	rs8102683[0 copy]	CNV	3.8×10^{-42}	$\beta = -4.00$
				rs11878604[C]	Intergenic	9.7×10^{-30}	$\beta = -2.69$

This table focuses on results achieving genome-wide significance (GWS). We used the significance threshold of 5×10^{-8}. The most significant GWAS finding from different studies for any specific variant is given. If numerous tightly mapped markers showed GWS in one study, only the most significant one is provided. Variant positions are based on NCBI Build 37/hg19. For many studies, it was not possible to extract the exact sample size used for each locus, so the sizes above are approximate. "Effect sizes" refers to beta coefficients for CPD and NMR and odds ratios for smoking initiation and cessation

CNV copy number variation, *CPD* cigarettes smoked per day, *FTND* dichotomized Fagerström Test for Nicotine Dependence: scores ≥4 vs. < 4, *NA* not available, *NMR* nicotine metabolite ratio, *OR* odds ratio; smoking cessation, whether regular smokers had quit at the time of interview; smoking initiation, ever vs. never began smoking

five variants in this region reached genome-wide significance in five GWAS and meta-GWAS (David et al. 2012; Liu et al. 2010; TAG 2010; Thorgeirsson et al. 2008, 2010), among which four (rs1051730, rs16969968, rs64952308, and rs55853698) were found to be significant in Europeans, and one (rs2036527) was significantly associated with CPD in African-Americans (AAs). The SNPs rs1051730, rs16969968, and rs55853698 are close-tagging proxies (all pairwise $r^2 > 0.96$) (Liu et al. 2010), and rs2036527 is correlated with rs1051730 (David et al. 2012). All the r^2s reported in the main text were extracted from the original studies. Thus, these variants were predicted to either tag or potentially cause the principal risk for high smoking quantity attributable to the 15q25 locus, with approximately one CPD step increase for each risk allele (David et al. 2012; Liu et al. 2010; TAG 2010). Although the synonymous SNP rs1051730 (Y188Y) in *CHRNA3* showed the strongest association, the non-synonymous SNP rs16969968 (D398N) in *CHRNA5* and rs55853698 in the 5′-UTR of *CHRNA5* hold more promise of functional importance. In the European samples, conditional on rs16969968 or rs55853698, residual association was detected at rs588765, tagging high expression of *CHRNA5* and rs6495308 within *CHRNA3* as showing significant association with CPD unconditionally. Liu et al. (2010) discovered better model fitting when conditioning on rs55853698 and rs6495308 compared with rs16969968 and rs588765 using the Bayesian information criteria (BIC). Both rs588765 and rs6495308 were reported to be in low linkage disequilibrium (LD) with each other ($r^2 = 0.21$) and both to be in only modest LD with the principal SNPs (maximum $r^2 = 0.47$) in subjects of European ancestry (Liu et al. 2010). However, in the AA samples, no second association signal was detected in this region after conditioning on rs2036527, suggesting that rs20356527 and correlated SNPs define a single common haplotype in populations of African ancestry (David et al. 2012). At the same time, the finding of importance of this gene cluster has been replicated by candidate gene association studies in persons of Asian ancestry (Chen et al. 2012c; Li et al. 2010b) and different ND phenotype-cotinine concentrations (Keskitalo et al. 2009), neural responses (Nees et al. 2013), smoking cessation successes (Bergen et al. 2013; Chen et al. 2012b; Munafo et al. 2011b), ages at initiation (Schlaepfer et al. 2008), and CPD during pregnancy (Freathy et al. 2009). The two most replicated variants in candidate gene association studies, rs16969968 and rs1051730, are consistent with the GWAS results. Please refer to Table 10.2 for details.

The three GWS SNPs on chromosome 8p11 in samples of African and European ancestries – rs13280604, rs6474412, and rs1451240 – are in perfect LD with each other (Rice et al. 2012; Thorgeirsson et al. 2010) and also with a variant (rs13277254) suggestively associated with the ND status of smokers in the first ND GWAS (Bierut et al. 2007). As noted by Rice et al. (2012), although the dichotomized FTND appeared to have an equivalent relation with rs1451240 across ethnicities, the relation between this SNP and CPD was much weaker in AAs than in European Americans (EAs). The other two SNPs were both significantly associated with CPD in Europeans (Thorgeirsson et al. 2010). These associated SNPs are either intergenic or intronic, which may tag causal variation(s) within the LD block that con-

tains *CHRNB3* and *CHRNA6* or regulate the expression of the two genes directly. Significant association of variants in *CHRNB3* and *CHRNA6* with ND was confirmed in eight candidate gene association studies with diverse population ancestries and smoking traits (Table 10.2) (Bar-Shira et al. 2014; Chen et al. 2012a; Cui et al. 2013; Culverhouse et al. 2014; Hoft et al. 2009; Lee et al. 2013; Saccone et al. 2009; Zeiger et al. 2008). Cui et al. (2013) obtained a close to GWS meta-*P* value for an upstream variant of *CHRNB3* (rs4736835) in a candidate gene association study of 22,654 subjects with African, European, and East Asian ancestries.

The last region detected by more than one GWAS or meta-GWAS is on chromosome 19q13.2 and includes genes such as *CYP2A6/A7/B6*, *EGLN2*, *RAB4B*, and *NUMBL*. Thorgeirsson et al. (2010) identified rs4105144 and rs7937 as significantly associated with CPD in European samples. These two SNPs were reported to be in LD with each other (r^2 = 0.32 and D' = 0.82 in the HapMap CEU samples). Rs4105144 was also in LD with *CYP2A6*2* (rs1801272; r^2 = 0.13 and D' = 1.0 in the HapMap CEU samples), which reduces CYP2A6's enzymatic activity (Thorgeirsson et al. 2010). The SNP identified by the Tobacco and Genetics Consortium (TAG 2010) (rs3733829) lies between these sites and was reported to show moderate LD with rs4105144 and rs7937. Besides association signals in samples with European ancestry, Kumasaka et al. (2012) found a copy number variant (CNV; rs8102683) with a strong effect on CPD ($\beta = -4.00$) in a Japanese population and another significantly associated SNP (rs11878604; $\beta = -2.69$) located 30 kb downstream of the *CYP2A6* gene after adjustment of the CNV. Rs8102683 shared a deletion region with other CNVs ranging from the 3′ end of the *CYP2A6* gene to the 3′ end of the *CYP2A7* gene; however, this common deletion was not significant in a European population (Kumasaka et al. 2012). Very recently, Loukola et al. (2015) conducted the first GWAS on nicotine metabolite ratio (NMR) and identified 719 GWS SNPs within this region. Strikingly, the significant *CYP2A6* variants explain a large fraction of the variance (as much as 31%) in NMR in their sample.

All the other signals reported by only one GWAS or meta-GWAS can be found in Table 10.3 and Fig. 10.1, among which a missense variant rs6265 in *BDNF* was significantly associated with smoking initiation, and an intergenic variant rs3025343 close to *DBH* was implicated in smoking cessation (TAG 2010). It is worth noting that GWASs without GWS variant identification still render valuable information in determining susceptibility loci for ND. The first ND GWAS, performed by Bierut et al. (2007), nominated *NRXN1* in the development of ND, which was validated by a subsequent candidate gene association study (Nussbaum et al. 2008). By using a network-based genome-wide association approach, Vink et al. (2009) discovered susceptibility genes encoding groups of proteins, such as glutamate receptors, proteins involved in tyrosine kinase receptor signaling, transporters, and cell-adhesion molecules, many of which were confirmed in later candidate gene association studies (Beuten et al. 2006a; Ma et al. 2010).

5 Targeted Sequencing Studies on Smoking Addiction

As the “missing heritability” issue emerged in the genetics field, researchers suspected that much of it is attributable to genetic variants that are too rare to be detected by GWAS but may have relatively large effects on risk and thus are important to study using next-generation sequencing technologies (Cirulli and Goldstein 2010). Both population genetic theories and empirical studies of several complex traits suggest that rare alleles are enriched for functional and deleterious effects and thus are disproportionately represented among disease alleles (Sham and Purcell 2014).

For the field of ND genetics, rare variant investigation started with the nAChR subunit genes, which not only are biologically important but also have yielded the most replicable results in both GWASs and candidate gene association studies, as presented above. Wessel et al. (2010) first examined the contribution of common and rare variants in 11 nAChR genes to FTND in 448 EA smokers, which revealed significant effects of common and rare variants combined in *CHRNA5* and *CHRNB2*, as well as of rare variants only in *CHRNA4*. Xie et al. (2011) followed up on the *CHRNA4* finding by sequencing exon 5, where most of the nonsynonymous rare variants were detected, in 1000 ND cases and 1000 non-ND controls with equal numbers of EAs and AAs. They discovered that functional rare variants within *CHRNA4* may reduce ND risk. Also, Haller et al. (2012) detected protective effects of missense rare variants at conserved residues in *CHRNB4*. They examined in vitro the functional effects of the three major association signal contributors (i.e., T375I and T91I in *CHRNB4* and R37H in *CHRNA3*), finding that the minor alleles of those SNPs increased the cellular response to nicotine. The two rare variants in *CHRNB4* were confirmed to augment nicotine-mediated α3β4 nAChR currents in hippocampal neurons, as did a third variant, D447X, in the report of Slimak et al. (2014). The fourth SNP they analyzed, R348C, reduced nicotine currents. They also observed that habenular expression of the β4 gain-of-function allele T374I resulted in a strong aversion to nicotine in mice, whereas transduction of the β4 loss-of-function allele R348C failed to induce nicotine aversion. Later, Doyle et al. (2014) reported an interesting rare variant in *CHRNA5* that could result in nonsense-mediated decay of aberrant transcripts in 250 AA heavy smokers. And recently, Yang et al. (2015) performed a targeted sequencing study with the goal of determining both the individual and the cumulative effects of rare and common variants in 30 candidate genes implicated in ND. Rare variants in *NRXN1*, *CHRNA9*, *CHRNA2*, *NTRK2*, *GABBR2*, *GRIN3A*, *DNM1*, *NRXN2*, *NRXN3*, and *ARRB2* were found to be significantly associated with smoking status in 3088 AA samples, and a significant excess of rare variants exclusive to EA smokers was observed in *NRXN1*, *CHRNA9*, *TAS2R38*, *GRIN3A*, *DBH*, *ANKK1/DRD2*, *NRXN3*, and *CDH13*. The 18 genetic loci implicated in targeted sequencing studies are marked in Fig. 10.1.

6 Comparison of Susceptibility Loci for Smoking Addiction from Different Approaches

According to our literature search, 242 candidate gene association, 22 genome-wide linkages, 18 GWAS, and 5 targeted sequencing, making a total of 287 studies, have been conducted in the ND genetics field. As a summary and refining of the 286 ND genetic studies, we developed an ND genetic susceptibility map with 14 linkage regions and 47 unique loci of 60 susceptibility genes (Fig. 10.1).

Both genome-wide linkage and GWAS are considered "unbiased" exploratory approaches. By comparing their results, we found that only two GWS signals are within the nominated linkage peaks, which are *LOC100188947* and *BDNF* (TAG 2010). The other nine loci, including the three most replicable ones, are all outside of the linkage peaks, and the rest of the 12 linkage regions do not contain any GWS signal (Tables 10.1 and 10.2). This discrepancy might reflect the different natures of the two genome-wide approaches. Genome-wide linkage studies usually investigate sparse microsatellites segregated with the trait of interest in different families, whereas GWAS takes advantage of dense common variants in thousands of unrelated individuals. Because of the distinct characteristics of family and case-control samples and known locus heterogeneity for ND, we might not expect the same sets of susceptibility alleles to be detected by both approaches. The relatively large nominated linkage regions tagged by microsatellites may implicate common or rare variants or both within the region of interest, and it is generally believed that only common variants can be detected by GWAS. However, even if a linkage region is driven by common variants, we may not be able to locate it by GWAS because of the stringent *P* values applied for defining significance in that method. The presence of GWAS signals outside linkage peaks might also result from the lack of power for linkage studies to detect weak genetic effects exhibited by the loci involved in complex diseases compared with association studies (Risch and Merikangas 1996). As one can see, these unbiased approaches are powerful in marking areas in the genome; nevertheless, the areas they indicate are often large and may not be complete. In this case, hypothesis-driven studies are necessary tools, not only to scrutinize marked areas but also to explore promising false-negative results and biologically plausible targets.

Both candidate gene association and targeted sequencing studies serve this purpose. Candidate gene association studies replicated and extended 5 of the 11 GWAS results, i.e., *CHRNB3*/*A6*, *DBH*, *BDNF*, *CHRNA5*/*A3*/*B4*, and *EGLN2*/*CYP2A6*/*B6*. For the other 29 non-GWS candidate genetic loci, 20 and 7 were selected from within and close to linkage peaks, respectively, the exceptions being *NRXN1* and *DDC* (Table 10.2), which reminds us of the importance of examining suggestive results in GWAS (Bierut et al. 2007), the other two examples being *GRIN2B* and *NTRK2* (Vink et al. 2009), and biologically plausible genes separately. Although we have localized candidate genes within most of the nominated linkage regions, four peaks, on chromosomes 3q26–q27, 5q11.2–q14, 9p21–p24.1, and 17q24.3–q25.3, are still empty, suggesting there are novel susceptibility genes to be discovered in

the future. Overlaps and distinctions from the two unbiased approaches and the significant number of loci reproduced or proposed in candidate gene studies suggest that we have many more study targets with good statistical evidence besides the three most replicable GWAS loci. The fourth "immature" approach is also hypothesis driven and has verified the importance of rare variants in ND genetics (Haller et al. 2012; Wessel et al. 2010; Xie et al. 2011; Yang et al. 2014). Besides the demonstrated aggregate effects of rare variants in 12 genetic loci implicated in previous studies, biological candidates showing equivocal or no association beforehand were found to be significantly associated with ND-related phenotypes, such as *CHRNB2*, *CHRNA9*, *CHRNA2*, *NRXN2*, *NRXN3*, and *CDH13*, among which *CHRNA9* and *NRXN2* are within linkage regions (Yang et al. 2015). Thus, we believe whole-exome and whole-genome sequencing studies focusing on rare variants, as the third unbiased experimental approach, will reveal new susceptibility genes/variants and further dissect the existing targets.

It is worth noting that to replicate a genotype–phenotype association, every effort should be made to analyze phenotypes similar to those reported in the original study (Chanock et al. 2007). However, the ND genetics studies mentioned above involved a plethora of smoking-related phenotypes. In general, they can be classified into the following groups: (1) categorical variables along smoking trajectories, e.g., smoking initiation, status, and cessation, (2) ND assessed using DSM-IV or FTND, (3) smoking quantity such as CPD, and (4) endophenotypes such as NMR, cotinine, and CO concentrations or functional imaging results. At least two of the four phenotype groups have been used in genome-wide linkage studies (Table 10.1), candidate gene association studies (Table 10.2) and GWASs (Table 10.3). Because of the sample source and size requirement differences, DSM- or FTND-ascertained ND definitions were commonly used in linkage studies, whereas CPD was more often applied in GWAS. For candidate gene-association studies, more comprehensive smoking profiles usually were tested for association with positive results from unbiased studies as replication, or more importantly, extension using different phenotypes (see Table 10.2), because there is considerable evidence that the various smoking measures are not highly related to each other (Piper et al. 2006). Even for measures with relatively high correlation, such as FTND and CPD, the slight change of phenotype from FTND-based ND to CPD would change the results (Rice et al. 2012). Therefore, although several loci, such as *TTC12–ANKK1–DRD2*, *CHRNA5/A3/B4*, and *CYP2A6/B6*, showed associations with different phenotypes (Tables 10.2 and 10.3), we should not expect positive associations with one phenotype to be replicated in samples with other phenotypes. It is important to keep in mind that a small change in phenotype may expose previously undiscovered variants, which underlie different biological processes and may have specific roles in distinguishing phenotypes (Rice et al. 2012).

Additionally, gene–gene and gene–environment interactions are two pieces of information missing from the current map because of the small number of reported studies. We expect more results in these two areas will be published with the development of efficient algorithms and become important parts of the susceptibility map. It also is worth noting that half of the 48 ND loci are significantly associated

with alcohol-related phenotypes, and about 30% are involved in illicit drug dependence, suggesting that the 60 genes on the ND map are good candidates for addiction studies of other drugs as well.

7 Concluding Remarks

Technological advances enable the development of different experimental approaches. A genetic susceptibility map, as put together in this chapter, contains scientific evidence from diverse approaches and can serve as a draft of the "parts list" to be updated periodically until complete (Sullivan et al. 2012). We hope such an enumeration will catalyze an array of specific targeted and nuanced scientific studies, as suggested by Sullivan et al. (2012), e.g., calculating the heritability explained by the 47 genetic loci, replicating association signals currently inadequately supported, identifying causal variant(s) within each locus through expression data integration and functional characterization, elucidating biological mechanisms between the genotype and ND, exploring gene–gene and gene–environment interactions, understanding the part played by epigenetic modifications, developing and evaluating treatment prediction models, and so forth.

Although the sample size of candidate gene association studies has increased over the years, genetic power calculation and corresponding sample size ascertainment should always be a top priority before conducting genetic studies. Additionally, only 18% and 10% of the 287 studies investigated subjects with African and Asian ancestries, respectively, compared with 69% for European ancestry. Studying different populations is necessary to understand the genetic causes of ND in various ethnic groups. Concurrently, given the importance of rare variants suggested by targeted sequencing study results, thorough and well-powered genomic evaluations at the lower end of the allelic spectrum are needed. Whole-exome and whole-genome sequencing studies with enough statistical rigor would enable a substantial update of the ND genetic susceptibility map in the near future.

However, it is important to acknowledge that the genetic liability accounted for by each of the 47 loci is low, considering their respective effect sizes, which may also explain why they can be identified through one type of unbiased study but not another. Anticipating future studies on the predictive power of these loci cumulatively, we are inclined to project that the amount of heritability explained still will be limited, which renders the susceptibility map only a beginning. Furthermore, functional studies have been conducted for limited genetic variants with certain or uncertain smoking associations (Table 10.4). Nevertheless, the *TTC12–ANKK1–DRD2* cluster shows consistent association with smoking-related behaviors (see Table 10.2), and the function of the most prominent variation in this region, *Taq1A*, still is largely unknown. On the other hand, we have understood the molecular and neurobehavioral functional consequences of the *BDNF* Met66Val polymorphism (rs6265) for more than a decade (Egan et al. 2003), although its association with ND phenotypes is still relatively weak (Table 10.2). Combining the susceptibility map

Table 10.4 Functional studies of variations associated with smoking in the 47 ND susceptibility loci

Chr.	Gene	Experiment	Variation [effect allele]	Effect
1	*CHRNB2*	In vitro gene expression assay	rs2072658 [A]	Reduced expression
6	*OPRM1*	PET brain imaging	rs1799971 [G]	Binding potential and receptor availability change
8	*CHRNA2*	Electrophysiology assay	rs141072985	nAChR function change
			rs56344740	
			rs2472553	
	CHRNB3	In vitro gene expression assay	rs6474413 [C]	Reduced expression
		ChIP and in vitro gene expression assay	rs4950 [G]	Eliminated TF binding and reduced promoter activity
9	*DNM1*	In vitro gene expression assay	rs3003609 [T]	Reduced expression
11	*BDNF*	fMRI, ^{1}H–MRSI, and immunoenzyme assays	rs6265	Different brain activation, BDNF secretion, and subcellular distribution
	DRD4	fMRI	Exon 3 VNTR	Different brain activation
15	*CHRNA5/A3/B4*	Imaging	rs16969968 [A]	Brain circuit strength prediction
		Series of in vitro assays		Altered response to nicotine agonist
		Electrophysiology and FLEXstation		Lower Ca permeability and increased short-term desensitization
17	*SLC6A4*	In vitro gene expression assay	5-HTTLPR	Transcriptional efficiency and expression change
		In situ hybridization		
		SPECT imaging		
19	*CYP2A6/B6*			
20	*CHRNA4*	Electrophysiology assay	Exon 5 haplotype	Different receptor sensitivity
22	*COMT*	Enzyme activity assay	rs4680 [A]	Less enzyme activity

ChIP chromatin immunoprecipitation, *fMRI* functional magnetic resonance imaging, *^{1}H-MRSI* ^{1}H magnetic resonance spectroscopic imaging, *nAChR* nicotinic acetylcholine receptor, *PET* positron emission tomography, *SPECT* single-photon emission computed tomography

results with relevant functional annotations will facilitate identification of the variations bearing higher translational values (Ducci and Goldman 2012). All in all, this map empowers us to sift through existing accomplishments and ponder future research strategies, an approach that may serve as a useful tool for other complex diseases/traits also.

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Chapter 11
Contribution of Gene–Gene and Gene–Environment Interactions to Tobacco Smoking

Abstract Like any other complex trait, tobacco addiction is controlled by multiple genetic factors, with each having a relatively modest effect, and by environmental factors, as well as by both gene–gene (epistatic) and gene–environment interactions. As summarized extensively in other chapters of this book, significant efforts have been made to search for susceptibility genes and variants for addiction to tobacco smoking. However, these approaches are effective only for genes and variants with moderate to significant effects. The ability to identify susceptibility genes for smoking addiction and other psychiatric disorders has been improving but remains considerably limited by the presence of a diverse array of factors such as epistatic interaction, modest marginal contribution, variable expressivity, small samples, and heterogeneities. Among these factors, epistatic and gene–environment interactions are of greatest importance. This chapter provides an update on the methods used to detect epistatic effect and representative examples of detected gene–gene interactions influencing smoking and other addictions.

Keywords Epistasis · Gene–gene interaction · Gene–environment interaction · GMDR · *CHRNA4* · *CHRNB2* · nAChRs · *BDNF* · *NTRK2* · *GABBR1* · *GABBR2* · *CHRNA5/A3/B4* · *HTR3A* · *HTR3B* · *SLC6A4* · Smoking dependence · Nicotine dependence

1 Introduction to Methods for Identifying Gene–Gene Interaction

For complex human diseases, interactions among genetic loci have become increasingly recognized (Jung et al. 2009; Zuk et al. 2012). Recently reported examples of the presence of gene–gene interaction include genes for coronary artery disease, type 2 diabetes, Alzheimer's disease, schizophrenia, breast cancer, cervical cancer, autistic disorder, and smoking addiction, to name a few. Such interactive effects among genetic loci may exist without a significant main effect of any of them; in such cases, important genetic effects would have been missed if polymorphisms of the involved loci had not been modeled jointly (Jung et al. 2009). Furthermore, in many cases, interactive effects of multiple genetic loci could be larger than the

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main effects of the individual loci (Robson et al. 2004; Rodriguez et al. 2006; Williams et al. 2000).

To search for determinants of epistatic and gene–environment interactions, prodigious efforts have been expended. Several combinatorial approaches, such as the multifactor dimensionality reduction (MDR) technique (Ritchie et al. 2001), the combinatorial partitioning method (CPM) (Nelson et al. 2001), and the restricted partition method (RPM) (Culverhouse et al. 2004), are promising tools for detecting epistatic and gene–environment interactions. Since the original report, MDR has been applied by many research groups to detect interactions underlying a spectrum of complex disorders. However, these established methods have critical limitations that restrict their practical use. For example, none of them allows adjustments for covariates; MDR is applicable only to dichotomous phenotypes, and CPM and RPM cannot handle categorical phenotypes. To overcome the limitations of these combinatorial approaches and to meet research needs in determining epistatic and gene–environment interactions for complex phenotypes, we have developed a generalized MDR (GMDR) as well as a pedigree-based GMDR (PGMDR) for case-control (Lou et al. 2007) and family-based (Lou et al. 2008) studies, respectively, that permit adjustments for discrete and quantitative covariates and are applicable to both dichotomous and continuous phenotypes.

The MDR was developed to detect genetic interactions by exhaustively searching multilocus combinations (Motsinger-Reif et al. 2008; Ritchie et al. 2001). In MDR, k (e.g., $k = 3$) factors and their possible multifactor classes are represented in k-dimensional space. Each multifactor class in the space is labeled “high risk” if the cases-to-controls ratio meets or exceeds some threshold or “low risk” if that threshold is not exceeded, thus reducing the k-dimensional space to one dimension with two levels (low and high risk) (Moore 2003). The best k-locus model is then selected, and the model is evaluated against the test group, and testing accuracy is calculated. Pedigree-based generalized MDR, a new generalized MDR for pedigree data, is a nonparametric method based on the score of the generalized linear model, which permits adjustment for covariates and handling of both dichotomous and quantitative phenotypes (Lou et al. 2008). A key advantage of PGMDR is that the method can handle different pedigree structures and sizes simultaneously in the presence of various patterns of missing data.

2 Variants in *CHRNA4* and *CHRNB2* Interactively Impact ND

It has long been known that nAChR subunits α4 and β2 must join in order to form a functional α4β2-containing heteromeric nAChR in order to function, and biochemical studies have revealed that the α4β2-containing nAChR subtype makes up the majority of the high-affinity nicotine-binding sites in the brain (Flores et al. 1992) and that the genes for both subunits are upregulated during chronic nicotine

Table 11.1 Detected interaction models for SNPs in *CHRNA4*, *CHRNB2*, *BDNF*, and *NTRK2*

Gene pair	SNP(s) included in each interaction model	Prediction accuracy	Cross-validation consistency	Empirical *P* value
CHRNA4	rs2273504, rs2229959, rs2236196	0.565	6	0.007
CHRNB2	rs2072661, rs2072660			
CHRNA4	rs2229959, rs1044396	0.552	4	0.031
BDNF	rs2030324			
CHRNA4	rs2273505	0.578	9	<0.0001
NTRK2	rs4075274			
CHRNB2	rs3811450, rs2072661	0.541	6	0.068
BDNF	rs2030324			
CHRNB2	rs2072661	0.593	6	0.002
NTRK2	rs993315, rs729560, rs1187272, rs1122530, rs1078947, rs4075274			
BDNF	rs2030324	0.578	9	0.002
NTRK2	rs4075274			

exposure (Marks et al. 1992; Whiteaker et al. 1998). Furthermore, activation of *CHRNA4* is sufficient for nicotine-induced reward, tolerance, and sensitization (Tapper et al. 2004). Moreover, knockout (KO) mice for the $\alpha4$ or $\beta2$ (*CHRNB2*) subunit show no high-affinity binding sites in their brains and do not self-administer nicotine, indicating that the $\alpha4\beta2$ subtype plays a primary role in the reinforcing effects of the drug (Picciotto et al. 1998; Tapper et al. 2004). However, except for *CHRNA4*, which has been associated with smoking in several independent samples (Feng et al. 2004; Hancock et al. 2015; Hutchison et al. 2007; Li et al. 2005), most reported studies found no association of *CHRNB2* with ND in humans (Feng et al. 2004; Li et al. 2005; Lueders et al. 2002; Silverman et al. 2000).

By using the GMDR approach, significant interactions were found between the variants in *CHRNA4* and *CHRNB2* in affecting ND, but no significant interactions were found among these variants within each gene (Table 11.1). This is noteworthy in that no significant association of *CHRNB2* with ND has been detected in four independent studies, except that a significant association of rs2072658 and rs2072661 in the $3'$-untranslated region (UTR) of the gene with reduced risks for smoking initiation, ability to quit smoking, or an early response to nicotine was reported recently (Conti et al. 2008). The reason for failing to detect a significant association of *CHRNB2* with ND by itself may be a strong dependence of *CHRNB2* effects on specific *CHRNA4* variants or the small samples used in those studies with relatively small marginal effects of *CHRNB2* in their study populations. This indicates that there exists a significant interaction between variants of *CHRNA4* and *CHRNB2* in affecting ND. We thus conclude that *CHRNB2* has a significant effect on ND through interaction with *CHRNA4*. More importantly, detection of a significant interaction of *CHRNB2* with *CHRNA4* in humans provides a plausible explanation for the well-documented experimental evidence wherein KO mice that lack the

α4 or β2 subunit of nAChRs show no high-affinity binding sites in their brains and do not self-administer nicotine (Picciotto 2003).

3 Variants in *GABBR1* and *GABBR2* Interactively Impact ND

As shown in Chap. 4, genome-wide linkage scans of various smoking phenotypes have revealed several regions that likely harbor susceptibility loci for ND, particularly on chromosomes 9, 10, 11, and 17 (Li 2008). Of these reproducibly identified regions, that on chromosome 9 is of particular interest (Bergen et al. 1999; Bierut et al. 2004; Gelernter et al. 2007; Li et al. 2003, 2006). The first gene identified from this linkage region was G-protein-coupled receptor 51 (*GABBR2*), for which several SNPs were found to be significantly associated with ND in a Caucasian sample (Beuten et al. 2005).

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system, whose actions are mediated by both ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. The $GABA_B$ receptors are seven transmembrane G-protein-coupled proteins that are pharmacologically functional only as heterodimers consisting of both $GABA_{B1}$ and $GABA_{B2}$ subunits (Bettler et al. 2004). The GABA neurons are part of the mesolimbic dopamine system, critically important in mediating the reinforcing properties of drugs of abuse. The $GABA_B$ receptors, in particular, are responsible for dampening the reinforcing effects of dopamine resulting from natural reward. Additionally, the GABA system is diffusely expressed in the brain; therefore, areas other than the mesolimbic system may be partly responsible for its effects. Evidence from both animal and human studies supports the value of $GABA_B$ receptor agonists in the treatment of drug abuse. Specifically, in preclinical studies, baclofen, a $GABA_B$ agonist, promoted abstinence and decreased the use of several drugs of abuse, including nicotine (Cousins et al. 2002). Baclofen also has been effective in reducing cigarette smoking and has been reported to alter the sensory properties of cigarettes, reducing their desirability (Cousins et al. 2001).

Given that functional $GABA_B$ receptors consist of both $GABA_{B1}$ and $GABA_{B2}$ subunits, we conducted gene–gene interaction analysis of these two subunit genes in affecting ND. Significant interactions were detected between a synonymous SNP in the transmembrane domain of *GABBR1* and SNPs located in the intronic regions among exons encoding transmembrane and cytoplasmic domains of *GABBR2* (Table 11.2). These statistical gene-by-gene interactions are biologically relevant, as the subunits interact to form a complete functional receptor. Thus, the statistical interaction most likely represents the functional properties of these two subunits. Furthermore, we found that the majority of significant interactions exist within the *GABBR2* gene, suggesting a stronger association of ND with *GABBR2* polymorphisms compared with associations of ND with both *GABBR1* polymorphisms and

Table 11.2 Detected interaction models for SNPs in *GABBR1* and *GABBR2*

Sample	Gene	SNPs included in interaction model	ND measure	Prediction accuracy	Permutated *P* value
Pooled sample	*GABBR1*	rs29230	HSI/FTND	0.55	0.001
	GABBR2	rs7865648-rs585819			
	GABBR1	rs29230	FTND	0.52	0.05
	GABBR2	rs7865648-rs669095-rs585819			
EA sample	*GABBR1*	rs29230	FTND	0.56	0.02
	GABBR2	rs7865648-rs6478676-rs585819			

GABBR1-by-*GABBR2* interactive effects. However, it should be noted that the *GABBR2* SNPs included in the interaction models are located in the intronic regions among exons encoding transmembrane and cytoplasmic domains of the GABA$_{B2}$ subunit. Therefore, these polymorphisms do not affect the amino acid sequence of the transmembrane and cytoplasmic subunits unless there is a strong linkage disequilibrium (LD) with a causative variant in an exon. Nevertheless, it is possible they affect the structure of mature GABA$_{B2}$ mRNA through alternative splicing, resulting in altered GABA$_{B2}$ protein subunits. Although such a molecular mechanism has yet to be elaborated, the presence of six alternatively spliced mRNA variants for GABA$_{B2}$ (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/) strengthens the significance of functional SNPs in *GABBR2* intronic regions.

Together, our results provide the evidence for a direct association of ND with *GABBR2* polymorphisms and an indirect, less significant, association with *GABBR1* polymorphisms. The involvement of the GABA$_{B}$ receptor in ND has been reported in many studies using animal models (Bettler et al. 2004), including a recently reported genetic study on zebra fish applying a nicotine behavioral assay in a forward screening of genes mutated through gene-breaking transposon mutagenesis (Petzold et al. 2009).

4 Variants in *BDNF* and *NTRK2* Interactively Impact ND

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, which are related to the canonical nerve growth factor and are found in the brain and the periphery. BDNF acts on certain neurons of the central nervous system (CNS) and the peripheral nervous system, helping to support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses. BDNF must act through its high-affinity receptor NTRK2 in order to support the survival and growth of diverse neuronal populations and influence the form and function of chemical synapses (Bramham and Messaoudi 2005). Furthermore, nicotine modulates the expression of *BDNF* and *NTRK2* at both the RNA and protein levels, indicating that nicotine regulates the BDNF/TrkB signaling pathway (Serres and Carney 2006; Sun et al. 2007; Yamada and Nabeshima 2003).

Although the biological interaction of *BDNF* with *NTRK2* has been established experimentally using *in vitro* and animal models, there is no report demonstrating the presence of gene–gene interactions between these two. As shown in Table 11.1, we detected highly significant interactive genetic effects on ND for the gene pair *BDNF* and *NTRK2* (with prediction accuracy ranging from 0.565 to 0.593; empirical *P* values <0.01 for all these pairs). To determine whether interaction analysis between each gene pair yields a better model than a single-gene approach, we also performed interaction analysis on the SNP(s) included in the best interaction model for each gene pair. A comparison of the predictive accuracy and empirical *P* value of each gene pair and the corresponding individual gene (with prediction accuracy from 0.494 to 0.564; empirical *P* value 0.020–0.454) further confirmed our finding that significant gene–gene interaction exists among these pairs in affecting ND.

Taken together, these findings demonstrate that *BDNF* interacts with *NTRK2* to contribute to ND by biological interactive mechanisms that have been demonstrated experimentally. This provides an example of how traditional analysis may fail to identify important risk genes and thus that the use of a validated detection strategy for interactions is warranted.

5 Variants in *CHRNA5/A3/B4* Gene Cluster Interactively Impact ND

The psychopharmacologic effects of nicotine are mediated primarily by functionally diverse neuronal nAChRs, a family of ligand-gated ion channels widely distributed in the brain. These nAChRs are involved in numerous physiological functions both in the brain and in the periphery (Gotti and Clementi 2004). To date, 12 neuronal nAChR subunits have been identified, consisting of nine α (α2–α10) and three β (β2–β4) subunits. The human genes for all of these subunits except α8 have been cloned (Graham et al. 2002). The 11 nAChR subunit genes are located on chromosomes 1, 4, 8, 11, 15, and 20, with *CHRNA5*, *CHRNA3*, and *CHRNB4* in a cluster on chromosome 15q24 (Raimondi et al. 1992). *CHRNA3* and *CHRNA5* are located in a tail-to-tail configuration on opposite DNA strands and share some of their 3′-UTR (Duga et al. 2001). Similarly, *CHRNB3* and *CHRNA6* are in a cluster on chromosome 8p11. The clustered arrangement of *CHRNA5/A3/B4* and *CHRNB3/A6* could affect the control of the expression of these genes (Flora et al. 2000; Xu et al. 2006).

Several subunit genes have been investigated for association with ND and other smoking-related behaviors in human subjects (see Chaps. 5 and 6 for further information and Lessov-Schlaggar et al. 2008; Li and Burmeister 2009 for reviews). In a recent study, Saccone et al. (2007) reported associations of multiple SNPs in the *CHRNA5/A3/B4* cluster with ND. However, the significance of these results did not survive correction for multiple testing. Since then, several genome-wide and candidate gene-based association studies provided further evidence for the association of

Table 11.3 Detected best interactive models for variants in *CHRNA5/A3/B4* cluster with ND in the AA and pooled sample

Sample	No. of loci	Best model	ND Measure	Prediction accuracy	*P* value
AA sample	4	*CHRNA5*: rs684513; rs615470	SQ	0.553	0.002
		CHRNA3: rs1317286	HSI	0.543	0.016
		CHRNB4: rs12441088	FTND	0.531	0.041
	6	*CHRNA5*: rs684513, rs621849	SQ	0.546	0.005
		CHRNA3: rs578776; rs1317286; rs12914385	HSI	0.549	0.003
		CHRNB4: r12441088	FTND	0.529	0.044
Pooled sample	3	*CHRNA5*: rs621849	SQ	0.530	0.011
		CHRNA3: rs3743078	HSI	0.520	0.061
		CHRNB4: rs11637890	FTND	0.516	0.106

variants of the *CHRNA5/A3/B4* gene cluster with various nicotine-related behaviors (Berrettini et al. 2008; Bierut et al. 2008; Chen et al. 2009; Greenbaum et al. 2006; Saccone et al. 2007; Schlaepfer et al. 2008; Sherva et al. 2008; Weiss et al. 2008).

Given that all nAChR subunits except α7 must assemble under appropriate conditions to become functional receptors, we conducted gene–gene interaction analysis on the variants in the *CHRNA5/A3/B4* cluster, which revealed significant interaction among some variants (Table 11.3). For example, in a Korea male sample, some genetic variants exist in the region between rs7163730 in LOC123688 and rs11072793 in the intergene region downstream from the 5′ end of *CHRNB4* that are contributing to smoking initiation through gene–gene interactions (Li et al. 2010). This appears consistent with the findings from our recent study of the association of the region with ND in European American (EA) and African-American (AA) samples (Li et al. 2009).

6 Variants in the Serotonin System Interactively Impact ND

The 5-HT$_{3A}$ subunit exists as a homomeric structure in the CNS. When the 5-HT$_{3A}$ subunit combines with the 5-HT$_{3B}$ subunit, they form pharmacologically more potent 5-HT$_{3AB}$ heteropentameric receptor complexes, which are distributed throughout the limbic structures implicated in addiction (Davies et al. 1999; Dubin et al. 1999; Enoch et al. 2011). On the other hand, the serotonin transporter (5-HTT) is the only molecule known to regulate synaptic serotonin concentrations through reuptake into presynaptic nerve terminals. Thus, it self-modulates the availability of serotonin molecules for binding with the 5-HT$_{3AB}$ receptors.

Considering the biological and pharmacological functions of the three genes in regulating serotonin signaling, we performed an exhaustive search of all possible two- to five-locus interaction models among the 17 polymorphisms in *HTR3A*, *HTR3B*, and *SLC6A4* for their epistatic effect on three ND measures in the AA, EA,

Table 11.4 Detected interaction models for variants in *SLC6A4*, *HTR3A*, and *HTR3B*

Sample	SNP combination	ND measure	Test accuracy	Cross-Validation Consistency(CVC)	Permutated *P* value
EA	*HTR3A*: rs1062613, rs1150220;	SQ	0.5678	7	0.003
	HTR3B: rs1176744	HSI	0.5699	9	0.002
	SLC6A4: 5-HTTLPR, rs1042173	FTND	0.5703	10	0.002
AA	*HTR3A*: rs10160548	SQ	0.5500	10	0.005
	SLC6A4: 5-HTTLPR, rs1042173	HSI	0.5458	10	0.009
Pooled	*HTR3A*: rs1062613, rs10160548	SQ	0.5516	8	0.00051
	HTR3B: rs1176744	HSI	0.5547	8	0.00025
	SLC6A4: 5-HTTLPR, rs1042173	FTND	0.5479	10	0.00085

and pooled samples. As shown in Table 11.4, the best interaction model detected for each sample shows a significant genetic interaction effect on all three ND measures, with an empirical *P* value < 0.01, cross-validation consistency (CVC) of at least 7 of 10, and test accuracies (TA) >50% based on 10^6 permutation tests except for the model on the Fagerström Test for ND (FTND) in the AA sample, where the empirical *P* value is 0.057. Of the three samples, the epistatic effect of the best interaction model for the three ND measures in the pooled sample appeared to be the strongest, with an empirical *P* value of 0.00025–0.00085.

In the pooled AA and EA samples, an interaction model consisting of five loci in *HTR3A*, *HTR3B*, and *SLC6A4* showed significant epistatic effects on all the three ND measures. These loci are rs1062613 and rs10160548 in *HTR3A*, rs1176744 in *HTR3B*, and 5-HTTLPR and rs1042173 in *SLC6A4*. Interestingly, the minor allele frequencies (MAFs) of these five polymorphisms are high, with the lowest frequency being 0.211 for rs1042173 in *SLC6A4* in the AA sample and 0.244 for rs1062613 in *HTR3A* in the EA sample. Of them, three polymorphisms alter the expression of the RNA, protein, or both encoded by the respective genes (Niesler et al. 2001). For example, rs1062613 is a translation regulatory variant located in an open reading frame upstream of the translation initiation site of *HTR3A* mRNA (Niesler et al. 2001). The two polymorphisms in *SLC6A4* alter 5-HTT expression through transcription regulation for 5-HTTLPR and degradation of mRNA transcripts for rs1042173 (Heils et al. 1997, 1996; Seneviratne et al. 2009; Vallender et al. 2008). Of the remaining SNPs, rs10160548 is located in intron 6 near an intron–exon boundary. It thus may alter the expression of functional *HTR3A* transcripts through alternative splicing. The rs1176744 in *HTR3B* does not alter expression but substantially changes serotonergic signaling through altered gating kinetics of the 5-HT_{3AB} receptor complex (Krzywkowski et al. 2008).

By analyzing the AA and EA samples independently, we revealed slightly different interaction models for each ethnic sample. In the AAs, there is a significant

interactive effect of SNPs rs10160548 in *HTR3A* and 5-HTTLPR and rs1042173 in *SLC6A4* on all three ND measures. In the EA sample, the model contains three additional loci: rs1062613 and rs1150220 in *HTR3A* and rs1176744 in *HTR3B*. In previous studies by other research groups, SNP rs1062613 in *HTR3A* has been associated with several psychiatric disorders in individuals of European descent (Gatt et al. 2010; Walstab et al. 2010). Yet, whether rs1062613 has ethnicity-specific *cis*-acting effects on the differential extents of translation of *HTR3A* in AAs and EAs remains to be characterized. However, inclusion of rs1062613 in the best interaction model in the pooled samples, with even stronger interaction effects than are seen in EAs only, argues against this possibility. The other *HTR3A* SNP, detected only in the EA sample, is rs1150220, which is moderately correlated with rs10160548 in both EAs and AAs ($r^2 = 0.42$ in AAs and $r^2 = 0.51$ in EAs) in an LD block located at the 3′ end of the *HTR3A* gene. The second main difference between the AA and EA samples is the absence of *HTR3B* rs1176744 in the best model for AAs. Although SNPs rs1176744 and 5-HTTLPR in *SLC6A4* are significantly associated with alcohol dependence in AAs (Enoch et al. 2011), we found no significant association of these two polymorphisms with ND in our AA, EA, or pooled samples, except for 5-HTTLPR, which showed a marginal association with FTND in the EA sample. However, our genetic interaction analysis demonstrated that the two polymorphisms in *SLC6A4* play an important role in ND through interactions with other SNPs in *HTR3A* and *HTR3B* in the AA, EA, and pooled samples.

7 Other Gene–Gene Interaction Examples Contributing to ND

Other examples of epistatic interaction are the demonstration of two genotype combinations, *COMT* Met/Met and *DAT* 10R or *COMT* Val/Val and *DAT* 9R, that are significantly associated with a blunted ventral striatal response (Yacubian et al. 2007). In considering a consistent relation between reduced reward sensitivity and addiction, these findings point to a potential genetic basis for vulnerability to addiction.

8 Contribution of Gene–Environment Interactions to Addiction

As mentioned earlier, the heritabilities of addictive disorders are in the range of 0.4–0.7 (Agrawal and Lynskey 2006; Goldman et al. 2005; Li and Burmeister 2009). This indicates there is a substantial environmental influence on these phenotypes, as is the case for other complex traits. However, unlike many other complex traits, environmental factors are a necessary component of all these addictive

disorders, regardless of one's genetic constitution. There is a broad agreement that environmental factors play a key role in the etiology of all addictive disorders; nevertheless, how to define and measure them and how to assess their interaction with genetic factors have remained unresolved for many complex traits, including addictive disorders (van der Zwaluw and Engels 2009). To some extent, determining the contribution of environment to addictions and gene–environment interactive effects can be as difficult and challenging as searching for genes for addictive disorders (Flint and Munafo 2008). Considering that genetic association studies have been characterized by non-replication and inconsistency, it has been suggested that research on addictions not only should not be restricted to genetic or environmental effects but also should include gene–environment interactions (Flint and Munafo 2008; van der Zwaluw and Engels 2009).

Unfortunately, the majority of studies in drug addiction have not accounted for gene–environment interactions except for a few studies in alcoholism (Ducci et al. 2008; Gelernter and Kranzler 2009; van der Zwaluw and Engels 2009). A classic example of a gene–environment interaction linked to psychopathology comes from a long-term prospective study by Caspi et al. (2002), showing that a functional polymorphism in *MAOA* was associated with later antisocial problems only if children were maltreated by their parents. However, several other research groups who tried to replicate this finding obtained mixed results (Gelernter and Kranzler 2009). Another example of gene–environment interaction study is related to the contribution of a polymorphism in *5-HTTLPR* to alcoholism. A meta-analysis linked the "S" allele of *5-HTTLPR* to increased alcohol consumption (Feinn et al. 2005). However, contradictory results have been reported in relation to the gene–environment interactions. For example, Nilsson et al. (2005) showed that the effects of *5-HTTLPR* genotypes on alcohol intoxication were particularly strong when adolescents reported poor family relations. In contrast, Dick et al. (2007) did not find an interaction between the *5-HTTLPR* genotype and stressful life events on alcohol dependence. Moreover, there is considerable evidence for gene–stress interaction for a functional polymorphism called *MAOA*-linked polymorphic region (MAOA-LPR), located in the promoter region of the gene, in influencing inappropriate control behaviors, including alcoholism and antisocial personality disorder (Ducci et al. 2008).

9 Concluding Remarks

Although it has long been known that gene–gene and gene–environment interactions contribute greatly to nicotine addiction and other psychiatric disorders, only a few gene–gene interactions have been reported. In this chapter, we presented several examples of detected gene–gene interactions in ND. This includes significant interactions among variants in *CHRNA4* and *CHRNB2*, *BDNF* and *NTRK2*, *GABAB1* and *GABAB2*, *CHRNA5/A3/B4* cluster and *SLC6A4*, and *HTR3A* and *HTR3B*. Importantly, most of these gene–gene interactions are well supported by biochemical and pharmacological studies.

However, as pointed out by Milne et al. (2008), replication of observed gene–gene interactions in additional independent samples is crucial. In particular, caution is needed during the replication because differences in LD between different study populations such as AAs and EAs may have important impacts on the detection of high-order gene–gene interactions. Even when significant interactive effects are observed in a replication study, caution is necessary in elucidating what exactly constitutes a replicated result and what is the biological meaning of such replication. Therefore, ideally, observed gene–gene interactions should not only be replicated from a statistical perspective but also should be experimentally validated from a biological perspective.

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Chapter 12
Identification of Biological Pathways Associated with Smoking Initiation/Progression, Nicotine Dependence, and Smoking Cessation

Abstract Twin and family studies reveal a significant genetic contribution to the risk of smoking initiation and progression (SI/P) and to ND and to the likelihood of smoking cessation (SC). Numerous genes have been implicated in these smoking-related behaviors, especially ND. However, no study has presented a comprehensive, systematic view of the genetic factors associated with these important smoking-related phenotypes. By searching the literature on these behaviors, we identified 16, 99, and 75 genes that have been associated with SI/P, ND, and SC, respectively. We then determined whether these genes were enriched in pathways important in the neuronal and brain functions underlying addiction. We identified 9, 21, and 13 pathways enriched in the genes associated with SI/P, ND, and SC, respectively. Among these pathways, four signaling pathways were common to all of the three smoking-related phenotypes: i.e., calcium, cAMP-mediated, dopamine receptor, and G-protein-coupled receptor. Further, serotonin receptor signaling and tryptophan metabolism pathways are shared by SI/P and ND; the tight junction signaling pathway is shared by SI/P and SC; and gap junction, neurotrophin/TRK signaling, synaptic long-term potentiation, and tyrosine metabolism are shared by ND and SC. Together, these findings demonstrate significant genetic overlap for these three related phenotypes. Although identification of susceptibility genes for smoking-related behaviors is still in an early stage, the approach utilized in this chapter has the potential to overcome the hurdles caused by factors such as genetic heterogeneity and small samples and thus should yield greater insights into the genetic mechanisms underlying these complex phenotypes.

Keywords Smoking initiation · Smoking persistence · Smoking cessation · Susceptibility genes · Pathways · Biological processes · Ingenuity Pathway Analysis · The Database for Annotation, Visualization, and Integrated Discovery · GeneTrail · Onto-Tools Pathway-Express · Association

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1 Introduction

Cigarette smoking is a complex behavior that includes a number of stages such as initiation, experimentation, regular use, dependence, cessation, and relapse. Although the initiation of tobacco use, the progression from initial use to smoking dependence, and the ability to quit smoking are undoubtedly affected by various environmental factors, twin, family, and adoption studies have provided strong evidence that genetics plays a substantial role in the etiology of these phenotypes (Goode et al. 2003; Lerman and Berrettini 2003; Lerman et al. 2007; Osler et al. 2001). Earlier studies revealed a considerable genetic contribution to the risk of smoking initiation and ND, as well as to the likelihood of successful smoking cessation.

So far, the majority of candidate gene-based association studies have focused on those genes that may predispose to addictive behavior by virtue of their effects on key neurotransmitter pathways (e.g., dopamine and serotonin) and genes that may affect the response to nicotine (e.g., nAChRs and nicotine metabolism). However, genetic studies have indicated that, for complex behaviors such as cigarette smoking, the individual differences can be attributed to hundreds of genes and their variants. Genes involved in different biological functions may act in concert to account for the risk of vulnerability to smoking behavior, with each gene having a moderate effect. Polymorphisms in related genes may cooperate in an additive or synergistic manner and modify the risk of smoking rather than act as sole determinants. Consistent with this belief, more and more genes have been found to be associated with smoking behavior over the past decades, especially during most recent years. Whereas some plausible candidate genes (e.g., nAChRs and dopamine signaling) have been reported, and the findings have been partially replicated, numerous genes involved in other biological processes and pathways also have been associated with different smoking behaviors. This is especially true as genome-wide association (GWA) study is being commonly used in genetic studies of complex traits such as smoking, and the underlying genetic factors can now be investigated in a high-throughput and more comprehensive approach. In this situation, a systematic approach that is able to reveal the biochemical processes underlying the genes associated with smoking behaviors will not only help us understand the relations of these genes but also provide further evidence of the validity of the individual gene-based association studies.

2 Identification of Genes Reported to Be Associated with Each Smoking Behavior

Contemporary genetic association studies of smoking behaviors are focused primarily on smoking initiation (SI), progression to smoking dependence and ND, which is assessed by various measures or scales such as DSM-IV, Fagerström Test for

Nicotine Dependence (FTND), Fagerström Tolerance Questionnaire (FTQ), or smoking quantity or SC. Many studies use more than one of these measures, as there is limited overlap in their assessments. Only a few studies have been conducted on SI and progression to ND. Considering the potential overlap of these two highly related behaviors, we combined them into the single category of smoking initiation and progression (SI/P).

The list of candidate genes for the three smoking-related phenotypes was constructed by searching all human genetic association studies deposited in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) using the queries for "(Smoking OR Tobacco Use Disorder) AND (Polymorphism OR Genotype OR Alleles) NOT (Neoplasms)." The abstracts of these articles were reviewed, and association studies of any of the three smoking-related behaviors were selected. We then narrowed our selection by focusing on papers reporting a significant association of one or more genes with any of the three phenotypes. To reduce the number of false-positive findings, the studies reporting negative or insignificant associations were excluded, although it is likely that some genes analyzed in these studies are in fact associated with the phenotypes we are interested in. The full reports of the selected publications were reviewed individually to ensure the conclusions were supported by the data. From these studies, genes reported to be associated with each phenotype were selected for the current study.

For SI/P, 16 genes were identified in 15 studies, all of which were performed at the individual gene level. Among them are five nAChR subunit genes, i.e., *CHRNA3*, *CHRNA5*, *CHRNA6*, *CHRNB3*, and *CHRNB4*, dopamine receptors D2 (*DRD2*) and D4 (*DRD4*), and one serotonin receptor (*HTR6*). The genes encoding transporters of dopamine (*DAT1* or *SLC6A3*) and serotonin (*5-HTT* or *SLC6A4*) were included. The other genes were those involving the functions related to nicotine or neurotransmitter metabolism/synthesis such as *COMT*, *CYP2A6*, and *TPH1*, signal transduction (e.g., *PTEN* and *RHOA*), or immune response (e.g., interleukin-8 [*IL8*]).

Regarding ND, there were 76 publications, including 73 focused on either a single or a few genes. In these papers, 63 genes were reported to be significantly associated with ND by the original authors. The other three studies were either on a genome-wide scale (Bierut et al. 2007; Uhl et al. 2007) or on hundreds of candidate genes (Saccone et al. 2007), and they nominated 41 genes. Collectively, 99 unique genes are on the final list. The most prominent are those encoding acetylcholine receptors (*CHRM1*, *CHRM5*, *CHRNA4*, *CHRNA5*, and *CHRNB2*), dopamine receptors (*DRD1*, *DRD2*, *DRD3*, and *DRD4*), GABA receptors (*GABRA2*, *GABRB2*, *GABARAP*, and *GABRA4*), serotonin receptors (*HTR1F* and *HTR2A*), and proteins involved in nicotine or neurotransmitter metabolism/synthesis (e.g., *CYP2A6*, *DBH*, *MAOA*, and *TPH1*).

For SC, 63 genes were nominated by a GWA study (Uhl et al. 2008) and 12 by 23 candidate gene-based association studies. These genes are involved in various signaling functions, such as dopamine receptor (*DRD2*, *DRD4*, and *SLC6A3*), glutamate receptor (*GRIK1*, *GRIK2*, *GRIN2A*, and *SLC1A2*), and calcium (e.g., *CACNA2D3*, *CACNB2*, *CDH13*, and *ITPR2*).

Among the genes associated with the three smoking phenotypes, five appeared on all three lists, i.e., *COMT*, *CYP2A6*, *DRD2*, *DRD4*, and *SLC6A3*. Another six genes, i.e., *CHRNA3*, *CHRNA5*, *CHRNB3*, *PTEN*, *SLC6A4*, and *TPH1*, were associated with both SI/P and ND. Ten genes, i.e., *A2BP1*, *ARRB2*, *CDH13*, *CHRNB2*, *CSMD1*, *CYP2B6*, *DBH*, *OPRM1*, *PRKG1*, and *PTPRD*, were associated with both ND and SC.

3 Enriched Biological Pathways Associated with Each Smoking-Related Phenotype

On the basis of the genes related to each smoking phenotype, enriched biochemical pathways were identified by Ingenuity Pathway Analysis (IPA; (http://www.ingenuity.com/), the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov), GeneTrail (http://genetrail.bioinf.uni-sb.de), Onto-Tools Pathway-Express (https://bioportal.bioontology.org/projects/Onto-Express), or some combination thereof.

For SI/P, the 16 genes were overrepresented in nine pathways defined in the IPA database ($P < 0.05$; Table 12.1). For five of these signaling pathways (calcium, dopamine receptor, serotonin receptor, cAMP-mediated, and G-protein-coupled receptor), the corresponding false-discovery rate (FDR) values were <0.05. For the other pathways (tryptophan metabolism, tight junction signaling, IL-8 signaling, and integrin signaling), they had slightly higher FDR values (0.085–0.116).

The IPA assigned 51 of the 99 genes associated with ND to 21 overrepresented pathways ($P < 0.05$; Table 12.2). Fourteen of these signalling pathways (e.g., dopamine receptor, cAMP-mediated, G-protein-coupled receptor, and serotonin receptor) had an FDR <0.05, and the other pathways (e.g., fatty acid metabolism and synaptic long-term potentiation) had an FDR <0.14.

Table 12.1 Pathways overrepresented by genes associated with smoking initiation/progression[a]

Pathway	*P* value	FDR	Genes included
Calcium signaling	2.24×10^{-6}	8.51×10^{-5}	*CHRNA3*, *CHRNA5*, *CHRNA6*, *CHRNB3*, *CHRNB4*
Dopamine receptor signaling	2.57×10^{-6}	4.88×10^{-5}	*COMT*, *DRD2*, *DRD4*, *SLC6A3*
Serotonin receptor signaling	1.12×10^{-5}	1.42×10^{-4}	*HTR6*, *SLC6A4*, *TPH1*
cAMP-mediated signaling	0.001	0.010	*DRD2*, *DRD4*, *HTR6*
G-protein-coupled receptor signaling	0.002	0.015	*DRD2*, *DRD4*, *HTR6*
Tryptophan metabolism	0.013	0.085	*CYP2A6*, *TPH1*
Tight junction signaling	0.018	0.099	*PTEN*, *RHOA*
IL-8 signaling	0.021	0.102	*IL8*, *RHOA*
Integrin signaling	0.028	0.116	*PTEN*, *RHOA*

[a]Pathways identified by IPA unless specified

Table 12.2 Pathways overrepresented by genes associated with nicotine dependence[a]

Pathway	*P* value	FDR	Genes included
Dopamine receptor signaling	1.58×10^{-13}	1.03×10^{-11}	*COMT*, *DDC*, *DRD1*, *DRD2*, *DRD3*, *DRD4*, *MAOA*, *MAOB*, *PPP1R1B*, *SLC18A2*, *SLC6A3*, *TH*
cAMP-mediated signaling	3.16×10^{-12}	1.03×10^{-10}	*ADRA2A*, *CHRM1*, *CHRM5*, *CREB1*, *DRD1*, *DRD2*, *DRD3*, *DRD4*, *GRM7*, *HTR1F*, *OPRM1*, *PDE1C*, *PDE4D*, *RAPGEF3*
G-protein-coupled receptor signaling	5.01×10^{-12}	1.03×10^{-10}	*ADRA2A*, *CHRM1*, *CHRM5*, *CREB1*, *DRD1*, *DRD2*, *DRD3*, *DRD4*, *GRM7*, *HTR1F*, *HTR2A*, *OPRM1*, *PDE1C*, *PDE4D*, *RAPGEF3*
Serotonin receptor signaling	6.31×10^{-11}	1.03×10^{-9}	*DDC*, *HTR2A*, *MAOA*, *MAOB*, *SLC18A2*, *SLC6A4*, *TPH1*, *TPH2*
Tryptophan metabolism	3.80×10^{-7}	4.94×10^{-6}	*CYP2A6*, *CYP2B6*, *CYP2D6*, *CYP2E1*, *DDC*, *MAOA*, *MAOB*, *TPH1*, *TPH2*
Calcium signaling	3.55×10^{-6}	3.53×10^{-5}	*CHRNA3*, *CHRNA4*, *CHRNA5*, *CHRNA7*, *CHRNB1*, *CHRNB2*, *CHRNB3*, *CREB1*, *TRPC7*
Tyrosine metabolism	3.80×10^{-6}	3.53×10^{-5}	*COMT*, *DBH*, *DDC*, *MAOA*, *MAOB*, *TH*
GABA receptor signaling	2.04×10^{-5}	1.66×10^{-4}	*DNM1*, *GABARAP*, *GABBR2*, *GABRA2*, *GABRA4*
Linoleic acid metabolism	4.37×10^{-4}	3.16×10^{-3}	*CYP2A6*, *CYP2B6*, *CYP2D6*, *CYP2E1*, *OC90*
Phenylalanine metabolism	1.66×10^{-3}	0.011	*DDC*, *MAOA*, *MAOB*
Arachidonic acid metabolism	2.09×10^{-3}	0.012	*CYP2A6*, *CYP2B6*, *CYP2D6*, *CYP2E1*, *OC90*
Metabolism of xenobiotics by cytochrome P450	2.57×10^{-3}	0.014	*CYP2A6*, *CYP2B6*, *CYP2D6*, *CYP2E1*, *EPHX1*
Histidine metabolism	3.55×10^{-3}	0.018	*DDC*, *MAOA*, *MAOB*
Neurotrophin/TRK signaling	0.011	0.049	*BDNF*, *CREB1*, *NTRK2*
LPS-/IL-1-mediated inhibition of RXR function	0.012	0.051	*ABCC4*, *CD14*, *CYP2A6*, *MAOA*, *MAOB*
Fatty acid metabolism	0.013	0.051	*CYP2A6*, *CYP2B6*, *CYP2D6*, *CYP2E1*
PXR/RXR activation	0.013	0.051	*CYP2A6*, *CYP2B6*, *NR3C1*
Synaptic long-term potentiation	0.039	0.140	*CREB1*, *GRM7*, *RAPGEF3*
Gap junction[b]	0.005	0.078	*DRD1*, *DRD2*, *HTR2A*, *PRKG1*
MAPK signaling pathway[b]	0.006	0.078	*ARRB1*, *ARRB2*, *BDNF*, *CD14*, *FGF14*, *NTRK2*
Regulation of actin cytoskeleton[b]	0.012	0.096	*ACTN2*, *CD14*, *CHRM1*, *CHRM5*, *FGF14*

[a]Pathways identified by IPA unless specified
[b]Pathway identified by Onto-Tools Pathway-Express

Table 12.3 Pathways overrepresented by genes associated with smoking cessation[a]

Pathway	P value	FDR	Genes included
Dopamine receptor signaling	2.29×10^{-6}	1.03×10^{-4}	*COMT*, *DRD2*, *FREQ*, *PPP2R2B*, *SLC6A3*
Glutamate receptor signaling	1.82×10^{-4}	4.10×10^{-3}	*GRIK1*, *GRIK2*, *GRIN2A*, *SLC1A2*
cAMP-mediated signaling	1.15×10^{-3}	0.017	*AKAP13*, *CREB5*, *DRD4*, *DRD2*, *OPRM1*
Calcium signaling	1.91×10^{-3}	0.022	*CHRNB2*, *CREB5*, *GRIK1*, *GRIN2A*, *ITPR2*
Circadian rhythm signaling	9.12×10^{-3}	0.082	*CREB5*, *GRIN2A*
Amyotrophic lateral sclerosis signaling	0.012	0.086	*GRIK2*, *GRIN2A*, *SLC1A2*
Synaptic long-term potentiation	0.017	0.096	*CREB5*, *GRIN2A*, *ITPR2*
G-protein-coupled receptor signaling	0.017	0.096	*CREB5*, *DRD2*, *DRD4*, *OPRM1*
Synaptic long-term depression	0.034	0.170	*ITPR2*, *PPP2R2B*, *PRKG1*
Tyrosine metabolism	0.037	0.170	*COMT*, *DBH*
Neurotrophin/TRK signaling	0.043	0.180	*CREB5*, *SORCS1*
Tight junction[b]	0.007	0.103	*CTNNA2*, *MAGI1*, *PARD3*, *PPP2R2B*
Gap junction[b]	0.022	0.171	*DRD2*, *ITPR2*, *PRKG1*

[a]Pathways identified by IPA unless specified
[b]Pathway identified by Onto-Tools Pathway-Express

For SC, 13 pathways were found to be enriched in 18 of the 75 genes associated with this phenotype ($P < 0.05$; Table 12.3). Four of the pathways (dopamine receptor signaling, glutamate receptor signaling, cAMP-mediated signaling, and calcium signaling) had an FDR <0.05, and the remaining pathways (e.g., synaptic long-term potentiation, G-protein-coupled receptor signaling, and synaptic long-term depression) had an FDR ranging from 0.082 to 0.18.

Of the pathways enriched in the genes associated with each smoking phenotype, four, i.e., calcium signaling, cAMP-mediated signaling, dopamine receptor signaling, and G-protein-coupled receptor signaling, were associated with all three smoking behaviors (Table 12.4). Two other enriched pathways (i.e., serotonin receptor signaling and tryptophan metabolism) were shared by SI/P and ND, and three (neurotrophin/TRK signaling, synaptic long-term potentiation, and tyrosine metabolism) were shared by ND and SC.

The enrichment of these pathways in multiple smoking phenotypes was consistent with the fact that synaptic transmission-related biological processes, such as nicotine-nAChR and dopamine signaling, were the key biochemical components underlying different smoking-related behaviors. This also implies that the genes involved in these three smoking phenotypes indeed overlap significantly. On the basis of these biochemical correlations, we present in Fig. 12.1 a schematic representation of the major pathways associated with the three phenotypes.

Table 12.4 Identified common and specific pathways for each smoking behavior category

Pathways	Smoking initiation and progression	Nicotine dependence	Smoking cessation
Calcium signaling	+	+	+
cAMP-mediated signaling	+	+	+
Dopamine receptor signaling	+	+	+
G-protein-coupled receptor signaling	+	+	+
Serotonin receptor signaling	+	+	–
Tryptophan metabolism	+	+	–
Gap junction	–	+	+
Neurotrophin/TRK signaling	–	+	+
Synaptic long-term potentiation	–	+	+
Tyrosine metabolism	–	+	+
Integrin signaling	+	–	–
Tight junction signaling	+	–	+
Arachidonic acid metabolism	–	+	–
Fatty acid metabolism	–	+	–
GABA receptor signaling	–	+	–
Histidine metabolism	–	+	–
Linoleic acid metabolism	–	+	–
LPS-/IL-1-mediated inhibition of RXR function	–	+	–
MAPK signaling pathway	–	+	–
Metabolism of xenobiotics by cytochrome P450	–	+	–
Phenylalanine metabolism	–	+	–
PXR/RXR activation	–	+	–
Regulation of actin cytoskeleton	–	+	–
Amyotrophic lateral sclerosis signaling	–	–	+
Circadian rhythm signaling	–	–	+
Glutamate receptor signaling	–	–	+
Synaptic long-term depression	–	–	+

4 Importance of Studying Susceptibility Genes for Smoking Behaviors at the Pathway Level

Over recent decades, much has been learned via animal or cell models about the molecular mechanisms underlying nicotine treatment. Numerous genes and pathways have been found to play a role, either directly or indirectly. However, it is less clear whether the same sets of genes and pathways are involved in tobacco dependence in humans. Epidemiological studies have shown that genetic factors are responsible for a significant portion of the risk for SI and ND and the likelihood of SC (Hamilton et al. 2006; Lerman and Berrettini 2003; Li et al. 2003; Mayhew et al. 2000; Sullivan and Kendler 1999). Moreover, significant genetic overlaps have been

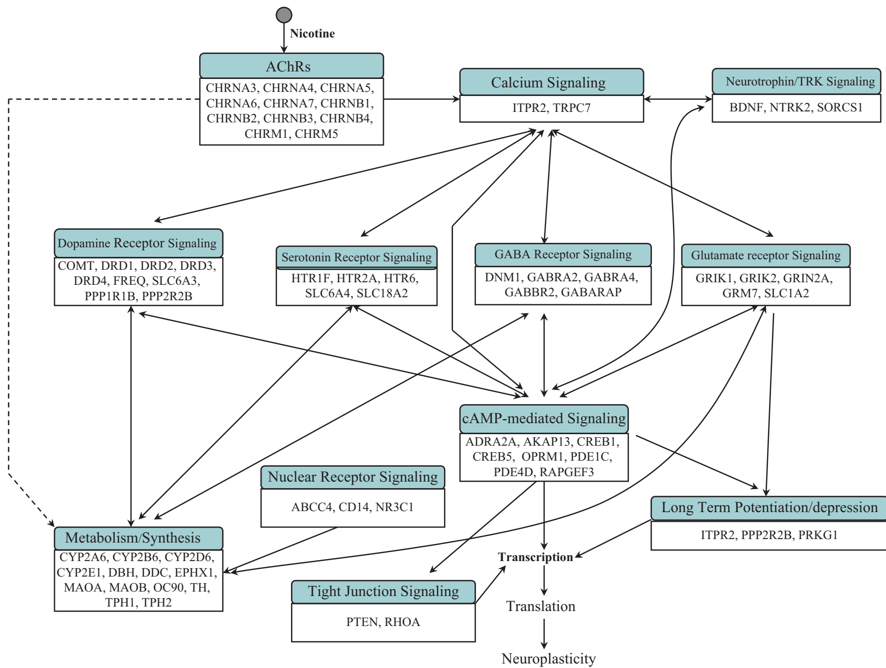

Fig. 12.1 Schematic representation of the genes and major pathways involved in smoking initiation/progression, dependence, or cessation. Genetic studies have indicated that tobacco smoking is a complex disorder. On the basis of the genes associated with SI/P, ND, and SC, we identified various enriched pathways corresponding to each phenotype. These pathways were then connected on the basis of their biological relations. Because of the overlap of many pathways among these three phenotypes, for the sake of simplicity, all pathways are shown together

identified among these three phenotypes (Ho and Tyndale 2007; Kendler et al. 1999; Maes et al. 2004). Identifying vulnerability genes for the three phenotypes, especially the biochemical pathways associated with them, not only will provide a systematic overview of the genetic factors underlying different smoking behaviors but also will be helpful in guiding the selection of potentially important genes for further analysis. With a thorough review of the genes contributing to the genetic risk of smoking behaviors, and a systematic search for gene networks using various pathway analysis tools, we provide a comprehensive view of the biochemical pathways involved in the three major smoking phenotypes (see Fig. 12.1 for details).

5 Single Gene-Based Association Analysis vs. Pathway Analysis

Although candidate gene-based association studies have provided much of our knowledge about factors contributing to smoking behavior, a systematic approach, as shown here, has significant advantages. For complex disorders such as tobacco

smoking, the presence of genetic heterogeneity and multiple interacting genes, each with a small to moderate effect, is considered to be the major hurdle in genetic association studies (Ho and Tyndale 2007; Lessov-Schlaggar et al. 2008). Numerous genetic factors have been implicated, but in many cases, these findings cannot be replicated in independent studies. At the same time, because of resource limitations, a significant proportion of reported genetic studies might not have sufficient population sizes or enough replication samples to reduce the rate of false-positive associations evoked by multiple testing. This is especially true for GWA studies, in which tens of thousands of SNPs can be analyzed simultaneously. A pathway approach, which takes account of the biochemical relevance of genes identified from association studies, not only can be more robust to potential false-positive results caused by factors such as low density of markers, small samples, different ethnicities, and heterogeneity within and between samples but also may yield a more comprehensive view of the genetic mechanism underlying smoking behaviors. Moreover, whereas in candidate gene-based association studies, the selection of targets focuses on some specific biological processes or pathways, the results from GWA studies appear to be more diverse. In such cases, pathway analysis becomes more necessary to detect the main biological themes from the genes involved in different functions. For example, Vink et al. (2009) identified 302 genes associated with SI and current smoking, but none of these genes were involved in classic targets, such as dopamine receptor signaling or nAChRs. Instead, they identified genes related to glutamate receptor signaling, tyrosine kinase signaling, and cell-adhesion proteins. In our analysis based on genes other than those reported by Vink et al., glutamate receptor signaling was enriched among the genes associated with SC, and TRK signaling was enriched in both ND and SC (see Tables 12.2 and 12.3 and Fig. 12.1). With the greater interest in conducting GWA studies for smoking behavior and other complex traits, a pathway approach will become more useful.

6 Potential Limitations of Pathway Analysis

There are several limitations of this type of study. First, the results depend entirely on the genes reported to be associated with each smoking phenotype of interest. Given that identification of susceptibility genes for each phenotype is an ongoing process, the pathways identified here should be treated in the same way. These pathways are only some of those that might be involved in the regulation of the three phenotypes. This is especially true for SI/P and SC, as significantly more genetic studies have been conducted on ND than on the other smoking phenotypes.

Second, we adopted the conclusions drawn by the original authors of each study. This means that some of our conclusions might be biased by those original reports because of their small sample size, the presence of heterogeneity, or the absence of correction for multiple testing. Initially, we tried to apply a general standard to all the reported studies but had to give up because different research groups conducted those studies over different times. It was challenging to redraw a conclusion from

those studies reported by other researchers. However, we do not think this will affect our results greatly, as we have included as many reports as we could get from the literature.

Third, for the sake of simplicity and increasing the number of genes included in each smoking phenotype, we classified more than 100 reports on smoking-related behaviors from different ethnic populations into three broad categories: i.e., SI/P, ND, and SC. This is certain to bring a heterogeneity issue to the three phenotypes of interest, especially SI/P and ND.

Fourth, the direction of an association is an important issue. For example, some variations are associated with a protective effect against SI or ND, whereas others increase the risk of such tendencies. Considering that the direction of association depends on the genetic variants under investigation for a given phenotype, we did not consider it in our current analyses. Because at this stage we are more interested in the genes and pathways potentially associated with smoking behaviors, focusing on the genes without considering the association directions will not create a serious problem. Also, to simplify the analysis and reduce the number of false-positive gene identifications, we did not include publications reporting negative or insignificant results. However, we realize that some genes from these studies may be among the factors associated with the smoking behaviors of interest. That they were not found to be associated is likely attributable to other factors such as the small sample or the presence of heterogeneity in the sample.

7 Description of Important Pathways Involved in Smoking Behaviors

We found that calcium signaling, dopamine receptor signaling, and cAMP-mediated signaling are the main pathways enriched in all three smoking phenotypes. The most prominent calcium signaling-related genes associated with each phenotype were nAChRs. By controlling the intracellular Ca^{2+} concentration, these ligand-gated cation channels play an important role in regulating various neuronal activities, including neurotransmitter release (Marshall et al. 1997; Wonnacott 1997). Transcription factors such as cAMP response element-binding proteins (CREBs) are crucial for the conversion of events at cell membranes to alterations in gene expression. Regulation of the activity of CREB by drugs of abuse or stress has a profound effect on an animal's responsiveness to emotional stimuli (Carlezon Jr. et al. 2005; Conti and Blendy 2004). The CREB function in neurons normally is regulated by glutamatergic and dopaminergic inputs (Dudman et al. 2003).

The mesolimbic dopamine pathway is believed to be one of the central pathways underlying addiction to various drugs (Nestler 2005). Genes in this pathway are among the major targets of association study for ND. Although this pathway is enriched in all the three smoking-related phenotypes, the genes associated with each phenotype are different. For SI/P, the genes reported in the literature, such as *COMT*,

DRD2, *DRD4*, and *SLC6A3*, are shared by ND and SC. For SC, two genes, *FREQ* and *PPP2R2B*, are uniquely detected. The *FREQ* protein (also known as neuronal calcium sensor 1; *NCS1*), a member of the neuronal calcium sensor family, has been implicated in the regulation of a wide range of neuronal functions such as membrane traffic, cell survival, ion channels, and receptor signaling (Burgoyne 2007). In mammalian cells, *FREQ* may couple the dopamine and calcium signaling pathways by direct interaction with *DRD2*, implying an important role in the regulation of dopaminergic signaling in normal and diseased brain (Kabbani et al. 2002). The interaction between variants of *DRD2* and *FREQ* significantly impacts the efficacy of nicotine replacement therapy (NRT) (Dahl et al. 2006). *PPP2R2B* encodes a brain-specific regulatory subunit of protein phosphatase 2A *(PP2A*) and gives rise to multiple splice variants in neurons (Dagda et al. 2003; Schmidt et al. 2002). The product of this gene is suggested to be localized in the outer mitochondrial membrane and involved in neuronal survival regulation through the mitochondrial fission/fusion balance (Dagda et al. 2008). A CAG-repeat expansion in a noncoding region of this gene is responsible for the neurodegenerative disorder spinocerebellar ataxia type 12 (*SCA12*) (Holmes et al. 1999). Although the dopamine receptor pathway plays an important role in all three smoking phenotypes, it is possible that different parts of this pathway are involved in each smoking behavior, with SI/P and ND having greater similarity than SC. Given the importance of this pathway to the development of drug addiction, more genes need to be verified to obtain a more specific picture of the role played in each phenotype.

Serotonin modulates dopamine release and has been implicated in nicotine reinforcement (see Chap. 9 for further information). Earlier study has shown that the serotonin concentration is increased by nicotine administration and decreased during drug withdrawal. Serotonin receptor signaling is enriched in the genes associated with SI/P and ND, but not SC, in our analysis. In several recent studies designed to investigate the association between genes from the serotonin receptor signaling pathway and SC, no positive result was obtained (Brody et al. 2005; David et al. 2007, 2008; Munafo et al. 2006; O'Gara et al. 2008). Similar to the serotonin receptor signaling pathway, tryptophan metabolism, the pathway involved in the biological synthesis of serotonin, is enriched in the genes associated with SI/P, but not in SC. Consistent with this result, to date, the clinical effects of serotonergic-based drugs in smoking cessation are largely negative (Fletcher et al. 2008). Although more study is needed, these results suggest that the genetic variants in serotonin receptor signaling and tryptophan metabolism pathways may be less important in smoking cessation.

Glutamate receptor signaling was found to be enriched in the genes associated with SC, but not the other two phenotypes. In a GWA study (Vink et al. 2009), multiple genes from the glutamate receptor signaling pathway were suggested to be associated with SI and current smoking. Similarly, the glutamate receptor signaling-related genes associated with SC were also identified by a GWA study (Uhl et al. 2008). The genes in this pathway associated with SC include *GRIK1*, *GRIK2*, *GRIN2A*, and *SLC1A2*, while *GRIN2A*, *GRIN2B*, *GRIK2*, and *GRM8* are associated with SI and current smoking (Vink et al. 2009). Another gene, *GRM7*, was suggested to be associated with ND in an earlier GWA study (Uhl et al. 2007). Taken

together, these results suggest that glutamate receptor signaling is involved in all three phenotypes of interest. Also, until now, most of the genes in this pathway were identified by GWA studies, demonstrating the great potential of studies of this type in identifying genetic variants related to smoking behavior.

The long-term potentiation (LTP) pathway also is enriched in genes associated with ND and SC, and the long-term depression (LTD) pathway was enriched in genes associated with SC. Repeated exposure of neurons to nicotine eventually leads to the modulation of the functioning of the neural circuits in which the neurons operate. Both LTP and LTD are thought to be critical mechanisms that contribute to such modifications in neuronal plasticity (Kauer 2004; Saal et al. 2003; Thomas and Malenka 2003). In the development of ND, the LTP and LTD pathways may be essential for the neurons to form new synapses and eliminate some unnecessary ones to adapt to a new environment. In the process of SC, these pathways may be invoked to interrupt some neuronal connections formed in the development of nicotine addiction in order to help the reward circuit return to normal. Until now, only a few genes related to LTP and LTD have been identified in association studies. Considering the importance of these pathways in ND development and SC, other genes associated with these processes represent potential targets for future studies of these phenotypes.

8 Concluding Remarks

These significantly overrepresented pathways suggest a view of neuronal responses in different conditions of nicotine–neuron interaction (Fig. 12.1). On binding by nicotine, the nAChRs open and cause the influx of Ca^{2+} and Na^{+} into the presynaptic neuron, which evokes depolarization, as well as activation of the Ca^{2+} signaling cascade. This cascade is directly related to the presynaptic release of neurotransmitters, including dopamine, serotonin, GABA, and glutamate, in different neurons. The neurotransmitters interact with their specific receptors, provoking a series of signaling pathways, such as cAMP-mediated and protein kinase C. With the regulation of these pathways, various physiological processes such as neuronal excitability and energy metabolism may be mediated. Variations in some of these genes may change the efficiency or function of the pathways and, eventually, the psychopathological phenotype. Although a significant number of genes associated with these pathways have been identified, our understanding of the genetic determinants of smoking is still in its early stages (Munafo and Johnstone 2008). It can be expected that as more genetic factors are identified, more detailed pathways and more comprehensive understanding of the mechanisms of human smoking behavior will be obtained.

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Chapter 13
Neuroproteomics and Its Applications in Research on Nicotine and Other Drugs of Abuse

Abstract The rapidly growing field of neuroproteomics is able to track changes in protein expression and the protein modifications underlying various physiological conditions, including the neural diseases related to drug addiction. Thus, it presents great promise in characterizing protein function, biochemical pathways, and networks to understand the mechanisms of drug dependence. In this chapter, we first provide an overview of proteomics technologies and the bioinformatics tools available to analyze the data. Then we summarize the recent applications of proteomics to profile the protein expression pattern in animal or human brain tissues after the administration of nicotine, alcohol, amphetamine, butorphanol, cocaine, and morphine. By comparing the protein expression profiles in response to chronic nicotine exposure with those appearing in response to treatment with other drugs of abuse, we identified three biological processes that appear to be regulated by multiple drugs of abuse: energy metabolism, oxidative stress response, and protein degradation and modification. Such similarity indicates that despite the obvious differences among their chemical properties and the receptors with which they interact, different substances of abuse may cause some similar changes in cellular activities and biological processes in neurons.

Keywords Neuroproteomics · Proteomics · Addiction · Pathways · Amygdala · Nucleus accumbens · Prefrontal cortex · Striatum · Ventral tegmental area · Nicotine · Alcohol · Amphetamine · Butorphanol · Cocaine · Morphine · Parkinson's disease · Alzheimer's disease

1 Introduction

Recent advances in genomics technology, along with bioinformatics development, are making it possible to analyze simultaneously the entire complement of genes expressed in a particular cell line or tissue. These technical advances have facilitated the identification and characterization of the three major genetic units: the genome, the transcriptome, and the proteome. The genome is the entire set of genes encoded by the DNA of an organism. The transcriptome consists of the entire complement of mRNA transcripts, and the proteome is the ensemble of protein forms expressed in

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a biological sample at a given time and in a particular situation. Both the transcriptome and the proteome differ from cell to cell and fluctuate in response to different physiological signals, including developmental cues, stress, drugs, changes in the extracellular environment, and disease.

In transcriptome research, the development of large-scale microarray technology allows comparison of the expression profiles of thousands of genes simultaneously. It is apparent that high-throughput techniques provide an efficient way to map complex biological pathways and to identify novel genes under an experimental condition of interest. During recent years, numerous microarray studies have focused on the effects of nicotine and other addictive drugs (e.g., alcohol, cocaine, morphine) on gene expression profiles (Bahi and Dreyer 2005; Dunckley and Lukas 2003; Kerns et al. 2005; Konu et al. 2001, 2004; Li et al. 2002, 2004; McClung et al. 2005; Rhodes and Crabbe 2005; Zhang et al. 2001). There is no doubt that studying RNA expression changes in response to these addictive drugs has provided significant insight into the molecular mechanisms underlying the dependence-inducing properties of each substance and impacted drug abuse research greatly (Li et al. 2002; Pollock 2002; Rhodes and Crabbe 2005; Yuferov et al. 2005). However, as we all know, many pharmacological and physiological effects of nicotine and other drugs in humans and animals are not mediated at the level of RNA alone but also at the level of protein and/or posttranslation. Furthermore, a difference in mRNA expression may not be a reliable predictor of a difference in protein expression. Although there are numerous reports on the effect of addictive drugs on the regulation of an individual protein, there have been only limited studies dealing with the global protein expression pattern with a systematic analysis using a high-throughput proteomics approach.

The application of proteomics to the study of nicotine and other substances of abuse is all but unexplored. Taking nicotine as an example, this chemical is believed to be the primary component in tobacco that rewards habitual smoking. Both epidemiological and molecular studies imply that many genes and proteins respond to nicotine stimulation. Even though numerous studies have been conducted to investigate how a gene or protein of interest is modulated by nicotine, using both in vivo and in vitro systems, only a limited number of systematic studies have been reported on gene expression profiles during chronic exposure to nicotine. Therefore, the mechanisms underlying the effects of nicotine in the brain are largely unknown, although it is generally believed that these effects are realized through the regulation of RNA and protein expression.

Proteomics has emerged in the last few years as a multidisciplinary technology-driven science concerned with systematic, large-scale analysis of the structure, function, and amount of the many proteins in a biological system. Although the term "proteomics" is only a few years old, its root goes back to the 1980s, when the usual methods of protein identification were immunoblotting and co-migration with known purified proteins in one-dimensional gel electrophoresis (Thrift et al. 1986). So far, two types of approaches have been employed in proteomics research to characterize proteins in large-scale production in the sample of interest: two-dimensional

(2D) gel electrophoresis/mass spectrometry (MS) (Gerner et al. 2000; Kanamoto et al. 2002; Lewis et al. 2000; Predic et al. 2002) and protein arrays (Haab et al. 2001; Miller et al. 2003; Nielsen et al. 2003; Sreekumar et al. 2001).

Broadly speaking, proteomics has four main objectives: (1) to identify all the proteins in a proteome, creating a catalogue of information; (2) to analyze differential protein expression associated with a disease, different cell states, or different treatments; (3) to characterize proteins by discovering their function, cellular location, posttranslational modifications (PTMs), etc.; and (4) to describe and understand protein interaction networks.

2 Protein Dynamics and Complexity in Substance Abuse Research

The rapidly evolving field of proteomics is directed toward providing a comprehensive view of the characteristics and activity of all cellular proteins. The proteome is clearly more complicated than the genome, as a single gene can encode multiple forms of a protein. This variable expression can result from alternative splicing of the mRNA transcript, use of alternative translational start or stop sites, and frameshifting, during which a different set of triplet codons is translated in the mRNA.

A surprising result of the genome projects for human, mice, and rats was the small number of genes in mammalian genomes. At present, between 20,000 and 25,000 protein-coding genes are predicted in the human genome, which represent less than 2% of the total genome sequence. Recent expression analysis using tiling arrays and the comprehensive characterization of transcriptional start and stop sites added new facets to this apparently low degree of complexity (Claverie 2005; Mendes Soares and Valcarcel 2006). Analysis of 1,000,000 expressed sequences in the mouse revealed more than 181,047 individual transcripts, surpassing the number of predicted mouse genes by a factor of nearly 10 (Carninci et al. 2005). More than 56,000 of the transcripts code for proteins, including previously undetected ones. At least 65% of the transcriptional units were modified by splicing, and numerous new splice variants were detected.

The highly dynamic proteome will require similarly dynamic quantitative models of protein pathways to capture an integrated cellular response to a substance. An expressed protein is in balance between its synthesis and breakdown rates, and proteomic changes can be homeostatic attempts to maintain normal physiologic function through altered protein expression and PTMs in response to a stimulus. A cellular phenotype involves several dynamic processes influenced by environmental signals. Transitions in the transcriptome and proteome after substance abuse involve several modification steps that include transcriptional cues and posttranslational processes.

The complexity of the cellular proteome, depicted in its temporal and spatial dynamic nature, results from the existence of multiple isoforms of many genes. An estimated ten protein isoforms can be generated from a single gene (Kim et al. 2004; Liebler 2002). Approximately 300 types of PTMs exist, such as amino- and carboxy-terminal cleavage, phosphorylation, glycosylation, and myristoylation (Garavelli 2004; Huber 2003). The human proteome contains more than 100,000 putative phosphorylation sites, and about 50% of all proteins supposedly are phosphorylated by one of the more than 500 known protein kinases (Manning et al. 2002). A high degree of complexity also is generated by glycosylation. Today, more than 2700 unique glycan structures are known. They arise from variation in the type, number, and position of individual sugar residues, the degree of branching, and the level of acetylation, methylation, phosphorylation, and sulfation (Cooper et al. 2003). It is estimated that a protein undergoes between 2 and 20 PTMs on average (Fountoulakis 2004). Taking into account the existence of more than 56,000 protein-coding transcripts and the complexity of the brain transcriptome, several hundred thousand protein species are to be expected in the CNS. After a drug treatment, the transcriptome is subject to a number of modifications, and newly modified mRNA transcripts give rise to different sets of proteins, which are subject to further modification, such as phosphorylation and dephosphorylation by kinases and phosphatases, proteolytic processing, acetylation, and glycosylation, among many others. Proteins also can be cross-linked by transglutaminase or conjugated to small tags such as ubiquitin or a small ubiquitin-like modifier (SUMO). Posttranslational modifications are important processes by which proteins acquire new functions or states in response to a specific cellular condition such as activation, turnover, downregulation, conformation, or localization (Morrison et al. 2002).

3 Recent Applications of Neuroproteomics in Research on Responses to Nicotine and Other Substances of Abuse

At present, a comprehensive analysis of whole-animal proteomes remains beyond our technology because of its extreme complexity. A more feasible approach is to focus on substructures, such as the brain and its components. During the past several years, proteomics has been used to profile the protein expression pattern in cultured neurons or different regions of the animal or human brain in response to a number of substances of abuse such as nicotine, amphetamine, alcohol, cocaine, and morphine. However, the application of proteomics technology to the study of a specific substance of abuse is still scarce; thus, it is useful to review most of the publications describing applications of proteomics techniques to research on substances of abuse such that we can have a good understanding of what we have learned from these applications in a comprehensive way.

3.1 Nicotine

Yeom et al. (2005) analyzed nicotine-associated protein expression in the striatum of rats. Seven proteins were found to be differentially regulated. Of these, zinc finger-binding protein-89 (ZBP-89), cyclic nucleotide phosphodiesterase (CNP1), and deoxyribonuclease 1-like 3 (DNASE1L3) were induced, whereas tandem pore domain halothane-inhibited potassium channel 2 (THIK2), brain-specific hyaluronan-binding protein (BRAL1), death effector domain-containing protein (DEDD), and brain-derived neurotrophic factor (BDNF) were suppressed. Although only one brain region was investigated, the study demonstrated the potential of proteomics to identify novel proteins associated with nicotine treatment. In a more comprehensive study reported by our laboratory (Hwang and Li 2006), we analyzed protein expression profiles for samples from five brain regions, i.e., the amygdala, nucleus accumbens (NA), prefrontal cortex (PFC), striatum, and ventral tegmental area (VTA) of rats that received nicotine for 7 days through osmotic pump infusion. Our study identified 14, 11, 19, 13, and 19 differentially expressed proteins in the amygdala, NA, PFC, striatum, and VTA, respectively. Of these, several proteins (e.g., dynamin 1, laminin receptors, aldolase A, SNAP-β, and *N*-ethylmaleimide-sensitive fusion protein) were differentially expressed in multiple brain regions. On the basis of Gene Ontology analysis, these differentially expressed proteins were grouped into various biological process categories, namely, energy metabolism, oxidative stress response, and protein modification and degradation.

3.2 Alcohol

Witzmann et al. (2003), Witzmann and Strother (2004) compared the protein expression differences in the hippocampus and NA of inbred alcohol-preferring and alcohol-nonpreferring rats. Their results revealed inherent expression differences of proteins in the two animal strains. Two proteins related to cellular signal transduction, i.e., cellular retinoic acid-binding protein 1 (CRABP1) and calmodulin-dependent protein kinase (CAMK1), were highly expressed in both brain regions but with the extent of expression in the alcohol-nonpreferring animals being much higher than in the alcohol-preferring animals. Also, the same research group (Bell et al. 2006) examined the protein expression changes in the amygdala and NA of inbred alcohol-preferring rats under different alcohol exposure conditions for more than 6 weeks. The differentially expressed proteins were grouped into various biological processes such as intracellular signal transduction, cytoskeleton, metabolism, cellular response to stress, and synaptic transmission. Similarly, Damodaran et al. (2006) found that alcohol treatment for 4 weeks altered protein expression in the brains of zebrafish. A total of eight proteins that were differentially expressed in response to alcohol was identified, which include voltage-dependent anion channel proteins (VDAC1 and VDAC2), heat-shock protein 70 (HSP70), alpha subunit of G_o

(GNAO1), and subunit A of the catalytic domain of H^+-transporting ATPase (ATP6V1A1). Moreover, Lewohl et al. (2004) and Alexander-Kaufman et al. (2006) applied a proteomic approach to tissue extracts of human brain obtained at autopsy. Lewohl et al. (2004) found 182 significant changes in protein expression in the alcoholic superior frontal cortex, among which were proteins related to antioxidant (e.g., peroxiredoxin 2, antioxidant protein 2), energy metabolism (e.g., pyruvate kinase M1 or M2), and heat-shock proteins (HSPA7 and HSPA8). Alexander-Kaufman et al. (2006) found that enzymes important for energy metabolism, such as creatine kinase chain B (CKB), NADH ubiquinone (MTND1), and fructose-bisphosphate aldolase C (ALDOC) were depressed in the brains of alcoholics.

3.3 Morphine

The diverse pharmacological and physiological effects of morphine are attributed mainly to its interaction with opioid receptors, members of the G-protein-coupled receptor family, to trigger multiple signal transduction pathways. Kim et al. (2005) analyzed the phosphotyrosyl (p-Tyr) proteins in the brains of morphine-dependent rats with the proteomic approach and found that 19 p-Tyr proteins were significantly upregulated in the frontal cortex, among which were signaling proteins such as 14-3-4 gamma (YWHAG), α-soluble NSF attachment protein (NAPA), and proteasome subunit β-type precursor. The expression of multiple enzymes is related to energy metabolism, such as pyruvate kinase (PK1), γ-enolase (ENO2), ALDOC, and malate dehydrogenase 2 (MDH2). Bierczynska-Krzysik et al. (2006) identified a few biomarkers associated with morphine addiction in rat brain, such as proteins related to protein modification and degradation, including 26S proteasome non-ATPase regulatory subunit 9 (PSMD9), ubiquinol-cytochrome C reductase iron-sulfur subunit (UQCRFS1), glutathione S-transferase P (GSTP1), superoxide dismutase (SOD1), and adenylate kinase (AK1). Li et al. (2006) examined protein expression in the NA of rats after chronic intermittent exposure to morphine. The major class of morphine-regulated proteins was found to be in the category of energy metabolism, such as enolase γ (ENO2), ATPase synthase β, and NADH-ubiquinone oxidoreductase. Prokai et al. (2005) analyzed the effect of chronic morphine exposure on the synaptic plasma-membrane subproteome in rats by the isotope-coded affinity tag (ICAT) method coupled with capillary reversed-phase liquid chromatography/electrospray ionization MS and tandem MS. Proteins involved in the regulation of the cell membrane potential such as Na^+/K^+ ATPase were found to be suppressed by morphine.

3.4 Amphetamine

Freeman et al. (2005) analyzed the proteomic profiles of the amphetamine self-administration transitional states in the hippocampus of rats. Compared with naïve, binge, and relapse groups, the expression of ALDOC, aspartate transaminase (GOT1), isovaleryl coenzyme a dehydrogenase (IVD), rab6-interacting protein 2 (RAB6IP2), enolase 1 α (ENO1), and heat-shock 60 kDa protein 1 (HSPD1) was reduced in the abstinent group, whereas the expression of β-actin (ACTB) and peroxiredoxin 2 (PRDX2) was induced in this group. Iwazaki et al. (2006) profiled protein expression in the striatum of rats treated by acute methamphetamine, a drug similar to amphetamine but with much more potent psychostimulant effects that is more harmful to the CNS. Proteins involved in energy metabolism, oxidative stress response, or signal transduction, including phosphoglycerate kinase 1 (PGK1), dihydrolipoamide dehydrogenase (DLD), rho GDP dissociation inhibitor α (ARHGDIA), PRDX2, and ubiquitin carboxy-terminal hydrolase L1 (UCHL1), were significantly regulated.

3.5 Cocaine

Tannu et al. (2007) analyzed the protein profile in the NA of cocaine overdose victims (CODs). Forty-seven proteins were found to be differentially expressed in the COD group and the control subjects. Some of the proteins increased in the COD group were β-tubulin, liprin-α3, and neuronal enolase, whereas the decreased proteins included parvalbumin, ATP synthase β-chain, and peroxiredoxin 2.

3.6 Butorphanol

Kim et al. (2004) analyzed the protein expression pattern in the frontal cortex of rats given chronic butorphanol tartrate, a mixed agonist–antagonist opioid analgesic agent. More than 60 p-Tyr proteins were regulated differently in the brains of drug-treated and control rats. The expression of most p-Tyr protein spots was increased in butorphanol-dependent rat brains compared with control samples. Fifty-three p-Tyr protein spots were identified as proteins involved in the cytoskeleton, cell metabolism, and cell signaling.

4 Proteomics Provides Insights into the Mechanisms Underlying Drug Addiction

Similar to other high-throughput approaches such as microarray technology, the true power of the proteomic approach is its ability to provide a comprehensive perspective on the protein family and pathways related to a specific condition such as exposure to a substance of abuse. As the two most widely used high-throughput technologies in the functional genomics field, both microarrays and proteomics can provide RNA/protein expression information on a genomic scale. Whereas microarray analysis can provide expression profiles at the mRNA level, proteomic analysis provides profiles at the protein level. The two approaches thus should be considered complementary. Proteomics provides an efficient way to map the expression of complex biological pathways and identifies proteins regulated under an experimental condition of interest. Furthermore, it enables comparison of the expression of the pathways or function-related proteins under different but related experimental conditions. This is especially valuable for the study of drug addiction, because such a comparison not only can help researchers understand the specificity of physiological effects of different drugs but also can provide insight into the similarity between the molecular and cellular mechanisms related to addiction to these drugs.

As stated earlier, by profiling the protein expression patterns in response to chronic nicotine treatment through a proteomic approach, we identified 63 differentially expressed proteins in five brain regions, i.e., amygdale, NA, PFC, ST, and VTA (Hwang and Li 2006). Of these unique proteins regulated by nicotine in one or more brain regions, 39 are differentially regulated by other drugs of abuse as well, namely, alcohol, amphetamine, butorphanol, cocaine, and morphine (Table 13.1). These commonly regulated proteins may be among the major molecules related to exposure to drugs of abuse and thus be of great interest. On the basis of their Gene Ontology information, these proteins can be grouped into multiple biological processes or pathways, particularly those related to energy metabolism, oxidative stress response, protein modification and degradation, signal transduction, and synaptic function. In this section, we concentrate our discussion on the first three of these biological processes as reported previously by our group (Hwang and Li 2006).

4.1 Energy Metabolism

In eukaryotic cells, most of the usable energy, ATP, is generated by aerobic respiration that converts carbohydrates, fats, and proteins into carbon dioxide and water. Four metabolic pathways are involved, i.e., glycolysis, which converts energy-containing molecules such as glucose into pyruvate; the pyruvate decarboxylation reaction, which converts pyruvate into acetyl-coenzyme A (CoA); the tricarboxylic acid cycle (TCA), which oxidizes acetyl-CoA into carbon dioxide and extracts energy primarily as the reduced electron carriers nicotinamide adenine dinucleotide

Table 13.1 Proteins modified by nicotine and one or more other substances of abuse

Protein & process	Function	Nicotine	Alcohol	Amphetamine/ Methamphetamine	Butorphanol	Cocaine	Morphine
Amino acid and protein metabolism							
GLN1	Gln synthetase	+					+ (Prokai et al. 2005)
GOT1	Glutamate oxaloacetate transaminase 1	+					+ (Prokai et al. 2005)
Energy metabolism							
ACO2	Aconitase 2, mitochondrial	+					+ (Prokai et al. 2005)
ALDOA	Aldolase, **fructose-bisphosphate**	+	+ (Bell et al. 2006; Park et al. 2004)		+ (Kim et al. 2004)		+ (Li et al. 2006)
ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, α, isoform 1	+	+ (Bell et al. 2006)	+ (Freeman et al. 2005)		+ (Tannu et al. 2007)	+ (Prokai et al. 2005)
ATP6V1A1	Similar to ATPase, H+ transporting, V1 subunit A, isoform 1	+	+ (Damodaran et al. 2006)		+ (Kim et al. 2004)		+ (Moron et al. 2007)
DLD	Dihydrolipoamide dehydrogenase	+					+ (Moron et al. 2007)
DLAT	Dihydrolipoamide acetyltransferase	+					+ (Prokai et al. 2005)
DRP2	Dihydropyrimidinase-related protein-2		+ (Alexander-Kaufman et al. 2006)		+ (Kim et al. 2004)		+ (Neasta et al. 2006)
ENO1	Enolase 1, α	+	+ (Lewohl et al. 2004)	+ (Freeman et al. 2005)	+ (Kim et al. 2004)	+ (Tannu et al. 2007)	

(continued)

Table 13.1 (continued)

Protein & process	Function	Nicotine	Alcohol	Amphetamine/ Methamphetamine	Butorphanol	Cocaine	Morphine
ENO2	Enolase 2, γ		+ (Bell et al. 2006)		+ (Kim et al. 2004)	+ (Tannu et al. 2007)	+ (Kim et al. 2005; Li et al. 2006)
GAPDH	Glyceraldehyde 3-phosphate-dehydrogenase		+ (Alexander-Kaufman et al. 2006; Bell et al. 2006)	+ (Freeman et al. 2005)	+ (Kim et al. 2004)		+ (Neasta et al. 2006; Prokai et al. 2005)
IDH3A	Isocitrate dehydrogenase 3 (NAD+), α	+				+ (Tannu et al. 2007)	+ (Prokai et al. 2005)
MOR1	Malate dehydrogenase, mitochondrial	+	+ (Bell et al. 2006)		+ (Kim et al. 2004)		+ (Prokai et al. 2005)
NDUFA10	NADH dehydrogenase 1, α, subcomplex 10-like protein	+					+ (Moron et al. 2007)
NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2	+	+ (Alexander-Kaufman et al. 2006; Bell et al. 2006)	+ (Freeman et al. 2005)			+ (Moron et al. 2007)
PDHB	Pyruvate dehydrogenase (lipoamide), β	+	+ (Lewohl et al. 2004)				
PK	M2 pyruvate kinase	+	+ (Lewohl et al. 2004)	(Iwazaki et al. 2006)	+ (Kim et al. 2004)		+ (Kim et al. 2005; Prokai et al. 2005)
TKT	Transketolase	+	+ (Alexander-Kaufman et al. 2006)				+ (Kim et al. 2005)
TPI1	Triosephosphate isomerase 1	+			+ (Kim et al. 2004)	+ (Hemby 2006)	+ (Li et al. 2006; Neasta et al. 2006; Prokai et al. 2005)
UQCRC1	Ubiquinol-cytochrome c reductase core protein I	+				+ (Tannu et al. 2007)	+ (Bierczynska-Krzysik et al. 2006)

VDAC1	Voltage-dependent anion channel 1	+	+ (Bell et al. 2006; Damodaran et al. 2006; Lewohl et al. 2004)				+ (Moron et al. 2007; Prokai et al. 2005)
Oxidative stress response							
AKR1B4	Aldo-keto reductase family 1, member B4	+				+ (Hemby 2006)	
GSTO1	Glutathione S-transferase omega 1	+				+ (Hemby 2006)	+ (Prokai et al. 2005)
GSTP1	Glutathione S-transferase, pi 1	+					+ (Bierczynska-Krzysik et al. 2006)
PRDX2	Peroxiredoxin 2	+	+ (Lewohl et al. 2004; Witzmann et al. 2003; Witzmann and Strother 2004)	+ (Freeman et al. 2005; Iwazaki et al. 2006)		+ (Tannu et al. 2007)	
Protein modification or degradation							
HSC70-ps1	Heat shock 70 kDa protein 8, variant	+	+ (Bell et al. 2006)				
HSPA8	Heat shock 70 kDa protein 8	+	+ (Bell et al. 2006; Lewohl et al. 2004)			+ (Hemby 2006)	+ (Moron et al. 2007)
HSPD1	Heat shock 60 kDa protein 1	+		+(Freeman et al. 2005)		+(Tannu et al. 2007)	+(Prokai et al. 2005)
PPIA	Peptidylprolyl isomerase A	+			+ (Kim et al. 2004)		+ (Prokai et al. 2005)
UCHL1	Ubiquitin carboxy-terminal hydrolase L1	+	+ (Alexander-Kaufman et al. 2006; Lewohl et al. 2004)	(Iwazaki et al. 2006)			+ (Li et al. 2006)
Signal transduction							
GNAO	GTP-binding protein alpha o	+	(Damodaran et al. 2006)		+ (Kim et al. 2004)		+ (Kim et al. 2005)

(continued)

Table 13.1 (continued)

Protein & process	Function	Nicotine	Alcohol	Amphetamine/ Methamphetamine	Butorphanol	Cocaine	Morphine
GNB1	Guanine nucleotide-binding protein, β-1 subunit	+					+ (Neasta et al. 2006)
PPP3CA	Protein phosphatase 3, catalytic subunit, α isoform	+				+ (Hemby 2006)	
YWHAZ	14-3-3 protein isoform zeta		+ (Bell et al. 2006)		+ (Kim et al. 2004)	+ (Tannu et al. 2007)	+ (Bierczynska-Krzysik et al. 2006; Moron et al. 2007)
Synaptic function							
DNM1	Dynamin 1	+					+ (Moron et al. 2007; Prokai et al. 2005)
NAPA	N-ethylmaleimide sensitive fusion protein attachment protein, α	+					+ (Kim et al. 2005; Neasta et al. 2006)
NAPB	Similar to Beta-soluble NSF attachment protein (SNAP-β)	+			+ (Kim et al. 2004)		
NSF	N-ethylmaleimide sensitive fusion protein	+	+ (Park et al. 2004)				+ (Prokai et al. 2005)
SEPT5	Septin 5	+					+ (Neasta et al. 2006)
STXBP1	Syntaxin binding protein 1	+	+ (Bell et al. 2006; Park et al. 2004)				+ (Moron et al. 2007; Prokai et al. 2005)
SYN2	Synapsin IIb	+					+ (Li et al. 2006)
Other							
ANXA5	Annexin A5	+	+ (Bell et al. 2006)				+ (Neasta et al. 2006)
CRYM	Crystallin, mu	+			+ (Kim et al. 2004)		+ (Kim et al. 2005)

(NADH) and flavine adenine dinucleotide (FAD) H2; and oxidative phosphorylation, which transfers electrons from NADH or $FADH_2$ to molecular oxygen and generates ATP via a series of protein complexes located on the inner mitochondrial membrane. Glycolysis and pyruvate decarboxylation take place in the cytoplasm, whereas the TCA cycle and oxidative phosphorylation take place in the matrix and inner membrane of mitochondria, respectively. This procedure is highly intricate and coupled, and each step is catalyzed by a series of enzymes or enzyme complexes.

The result of our proteomics analysis showed that proteins involved in aerobic respiration are regulated by chronic nicotine treatment in all the brain regions investigated (Hwang and Li 2006). For example, fructose-bisphosphate aldolase (ALDOA), an enzyme involved in glycolysis, is significantly regulated by nicotine in both the NA and the VTA. Interestingly, this protein also is modulated by ethanol (Bell et al. 2006; Park et al. 2004), butorphanol (Kim et al. 2004), and morphine (Li et al. 2006) (Table 13.1). Another member of the same family, ALDOC, is regulated by amphetamine (Freeman et al. 2005) and cocaine (Tannu et al. 2007). Several other enzymes related to energy generation, including ATP5G1 (ATP synthase: H^+ transporting, mitochondrial F1 complex, α subunit, isoform 1), ATP6V1A1 (similar to ATPase: H^+ transporting, V1 subunit A, isoform 1), ENO1, glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), malate dehydrogenase (MOR1), and triosephosphate isomerase 1 (TPI1), also are modulated by multiple substances of abuse.

Several proteins involved in glycolysis also are regulated by these drugs. For example, aldolases are ubiquitous enzymes that catalyze the reversible cleavage of fructose-bisphosphate to produce dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The vertebrate aldolase family consists of three isozymes (A, B, and C) (Berardini et al. 1997). Aldolase A is expressed predominantly in the muscle and brain, whereas aldolases B and C are expressed mainly in the liver (Cox 1994) and brain (Ahn et al. 1994), respectively. Aldolases play a fundamental role in the development and function of the nervous system and other tissues, and their abnormal expression has been linked to various diseases such as muscle weakness and premature muscle fatigue (DiMauro and Bruno 1998; Kreuder et al. 1996), stroke (Linke et al. 2006), Alzheimer's disease (Mor et al. 2005), and cancer (Asaka et al. 1994). Also, aldolases A and C have been suggested to regulate the stability of mRNA of light-neurofilament (Canete-Soler et al. 2005; Stefanizzi and Canete-Soler 2007), cytoskeletal components of large neurons, which are instrumental in maintaining the differential state. Enolases (e.g., ENO1 and ENO2), GAPDH, pyruvate dehydrogenase (lipoamide)-β (PDHB), M2 pyruvate kinase (PK), and triosephosphate isomerase 1 (TPI1) also are regulated by nicotine and other drugs of abuse.

The mitochondrial proteins modulated by nicotine and other substances of abuse include ATP5G1, ATP6V1A1, mitochondrial aconitase 2 (ACO2), isocitrate dehydrogenase 3 (NAD^+) alpha (IDH3A), malate dehydrogenase (MOR1), NADH dehydrogenase 1 alpha subcomplex 10-like protein (NDUFA10), and NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2). In mitochondria, ATP is synthesized using energy derived from a proton gradient by F_1F_o–ATP synthase (Berry 2005; Fillingame et al. 2003; Wilkens 2005), a multi-subunit complex

including ATP5G1. The regulation of ATP5G1 by nicotine and other substances of abuse implies a change in cellular ability to synthesize ATP. ACO2 is an iron-dependent metabolic enzyme that catalyzes the stereo-specific isomerization of citrate to isocitrate via cis-aconitate in the TCA cycle (Beinert and Kennedy 1993). IDH3A is a subunit of isocitrate dehydrogenase 3, which catalyzes the oxidative decarboxylation of isocitrate into alpha-ketoglutarate (LaPorte 1993). MOR1 catalyzes a reversible reaction that converts L-malate and NAD to oxaloacetate and NADH (Ball et al. 1994). All these enzymes play key roles in the TCA cycle. ACO2 also can undergo reversible citrate-dependent modulation in activity in response to pro-oxidants in mitochondria and is essential for maintaining the stability of mitochondrial DNA (Bulteau et al. 2004; Chen et al. 2005; Shadel 2005). Both NDUFA10 and NDUFS2 are subunits of NADH–ubiquinone oxidoreductase (complex I), the first multimeric complex of the respiratory chain that catalyzes NADH oxidation with concomitant ubiquinone reduction and proton ejection out of the mitochondria.

The regulation of proteins involved in energy metabolism by almost all the substances of abuse examined to date with neuroproteomics approaches indicates that the pathways related to energy generation are important in the response of neurons to treatment with each drug. Moreover, a number of mitochondrial proteins involved in energy metabolism are modulated by drugs of abuse, providing further evidence that the pathways of energy metabolism are highly modulated by nicotine and other substances of abuse. Actually, drugs of abuse can influence the activities of mitochondria. For example, nicotine treatment not only causes swelling of and structural damage to mitochondria (Jin and Roomans 1997; Onal et al. 2004; Zimmerman and McGeachie 1987) but also can regulate activities such as the protein turnover rate (Katyare and Shallom 1988), enzyme activity (Barbieri et al. 1989; Galvin et al. 1988; Xie et al. 2005), and generation of reactive oxygen species (ROS) (Cormier et al. 2001, 2003; Soto-Otero et al. 2002). Alcohol, amphetamine, cocaine, heroin, and morphine have similar effects on mitochondria (Boess et al. 2000; Brown and Yamamoto 2003; Cunha-Oliveira et al. 2006, 2007; Hajnoczky et al. 2005; Mastronicola et al. 2004; Oliveira et al. 2003; Ramachandran et al. 2001; Yuan and Acosta 1996, 2000). However, the mechanisms underlying the modulation of drug–mitochondria interactions are unclear. One of the plausible possibilities is that the drugs of abuse produce an altered environment in the CNS, forcing the neurons to adapt. The structure and function of mitochondria are then modulated in concert with the alteration of the extracellular and intracellular environment. The modulation of proteins in the mitochondria by the substances of abuse, as well as those related to energy generation and transduction in the cytoplasm, may create an inadequate supply of energy, which may lead to further changes in neurons. It also is likely that these drugs interact directly with specific pathways in mitochondria. Cormier et al. (2001, 2003) suggested that nicotine is an effective NADH competitor that inhibits mitochondrial NADH–ubiquinone reductase activity and significantly decreases the brain mitochondrial respiratory control ratio. Xie et al. (2005) proposed that nicotine can regulate the electron leak at the site of respiratory chain complex I on the mitochondrial membrane in an nAChRs-independent way.

Cunha-Oliveira et al. (2006) showed that both cocaine and amphetamine can interfere with the respiratory chain in mitochondria. The disturbance of the energy generation pathways has clearly indicated mitochondrial dysfunction caused by treatment with the drugs. Mitochondrial dysfunction would lead not only to energy deficiency but also to an increase in injurious ROS and reactive nitrogen species (RNS).

4.2 Oxidative Stress Response

Two members of an antioxidant family, peroxiredoxins 1 and 2 (PRDX1 and PRDX2), are downregulated by nicotine in the NA and PFC of rats, respectively (Hwang and Li 2006). PRDX2 is induced in the rat hippocampus at the abstinence stage after chronic amphetamine treatment (Rhee et al. 2005) and in the brain of human alcoholics (Mizusawa et al. 2000), whereas it is suppressed in the NA of CODs (Das et al. 2001; Ishii et al. 2000) and the striatum of acutely methamphetamine-treated rats (Bryk et al. 2000; Chae et al. 1994; Peshenko and Shichi 2001). This protein also is differentially expressed in the NA, hippocampus, and cortex of inbred alcohol-preferring and alcohol-nonpreferring rats (Butterfield et al. 1999; Fujii and Ikeda 2002). On the other hand, PRDX2 is a highly abundant cytosolic protein and a primary regulator of H_2O_2 generated by cell-surface receptors (Rhee et al. 2005). PRDX2 is expressed in neurons but not in glial cells and appears to be located in cells vulnerable to ischemic oxidative stress injury (Sarafian et al. 1999). PRDX2 provides an important function by protecting proteins and lipids against oxidative injury and regulates apoptosis by eliminating peroxides generated during metabolism (Kim et al. 2000; Netto et al. 1996; Yim et al. 1994).

Other members of this family also are modulated by drugs of abuse. For example, PRDX6 is upregulated in the frontal cortex region of rats after chronic butorphanol or morphine administration (Kim et al. 2004, 2005). It also is induced by cocaine in the NA of the human brain (Hemby 2006). Another member, PRDX5, is highly induced by acute methamphetamine exposure in the striatum of rats (Iwazaki et al. 2006).

Aldo-keto reductase family 1 member B4 (AKR1B4; also known as aldose reductase) is suppressed in the striatum of rats in response to nicotine treatment (Hwang and Li 2006). It also is downregulated by cocaine in the NA of the human brain (Hemby 2006). Aldose reductase is a member of the monomeric NADPH-dependent aldo-keto reductase family and participates in glucose metabolism and osmoregulation. It is believed to play a protective role against toxic aldehydes derived from lipid peroxidation and steroidogenesis that could affect cell growth/differentiation if accumulated (Lefrancois-Martinez et al. 2004).

Glutathione S-transferases (GSTs) are a family of phase II enzymes that utilize glutathione in reactions contributing to the transformation of a wide range of exogenous and endogenous compounds, including carcinogens, therapeutic drugs, and products of oxidative stress. The GSTs may prevent dopaminergic degeneration

through their direct antioxidant activity against various reactive metabolites of chemical toxicants produced by phase I enzyme metabolism (Harada et al. 2001; Miller et al. 2001; Santt et al. 2004). They may also serve a neuroprotective function by facilitating the elimination of endogenous toxins from the cell (Baez et al. 1997). Two members of this family, glutathione S-transferase π 1 (GSTP1) and glutathione S-transferase ω 1 (GSTO1), are suppressed by nicotine in the striatum of rats (Hwang and Li 2006). They also are downregulated by chronic morphine treatment in rat brain (Bierczynska-Krzysik et al. 2006; Prokai et al. 2005). GSTO1 is slightly upregulated in the brain of CODs (Hemby 2006).

The prooxidant property of addictive drugs has been one of the focuses of drug addiction study for a long time. Although nicotine has been suggested to be an antioxidant in the CNS because of its association with a decrease in the risk of certain neurodegenerative diseases such as Alzheimer's and Parkinson's (Newhouse et al. 1997), it also is able to induce oxidative stress in neurons and other cells, as evidenced by an increase in lipid peroxidation and free radicals and the inhibition of radical-detoxifying enzymes (Husain et al. 2001; Newman et al. 2002; Sener et al. 2005; Slotkin et al. 2005). Oxidative stress also is induced when the CNS is exposed to ethanol (Goodlett and Horn 2001; Goodlett et al. 2005; Sun and Sun 2001), morphine (Guzman et al. 2006; Ozmen et al. 2007), cocaine (Bashkatova et al. 2005, 2006; Poon et al. 2007), and amphetamine (Davidson et al. 2001; Yamamoto and Bankson 2005). Similar to the case of nicotine, oxidative stress evoked by these drugs can damage the viability of neurons and lead to apoptosis. The regulation of proteins related to antioxidants in the CNS by various addictive drugs, as revealed by proteomic studies, further suggests that oxidative stress may be one of the major physiological effects of these substances. Because of the presence of high proportions of polyunsaturated fatty acids and low concentrations of oxidant enzymes, the brain is particularly susceptible to oxidative stress (Sun and Sun 2001). Therefore, the presence of chronic and excessive oxidative stress in this organ can be destructive and may exacerbate the progression of neurodegenerative disorders.

Oxidative stress is caused by an imbalance in the prooxidant and antioxidant systems (Butterfield et al. 2006). Mitochondria are the primary source of intracellular ROS and RNS. In the process of oxidative phosphorylation, oxygen is reduced to water when transportable chemical energy, ATP, is generated. However, the reduction of oxygen can be incomplete, and ROS/RNS such as H_2O_2 is generated by the complexes in the electron transport chain located on the mitochondrial membrane (Brown and Yamamoto 2003). Normally, most of these oxidative species are cleared to maintain the redox status of mitochondria. Some of these oxidative substances also can diffuse across the outer membrane of mitochondria into the cytoplasm. The reactive species have dual actions from a biological point of view. On one hand, they may play roles in cellular defense and signal transduction when the concentration is low; on the other hand, they represent a threat of deleterious effects by oxidizing important structures and macromolecules when the concentration is high. Oxidative stress can evoke reversible or irreversible modification of macromolecules such as protein oxidation (Stadtman 2006), lipid peroxidation (Butterfield and Lauderback 2002), and DNA/RNA oxidation (Gabbita et al. 1998; Nunomura

et al. 1999) in neurons and consequently cause the dysfunction of these molecules and the loss of the reductive potential of the cells. To cope with overproduction of ROS, cells need to recruit antioxidant systems such as PRDX1 and PRDX2 to clean up the excess. However, the antioxidant system seems to be disrupted by drugs of abuse. The antioxidant proteins such as PRDX1 and PRDX2 are mitochondrial and cytoplasmic thioredoxin reductases, which are induced by accumulation of ROS, allowing cells to survive oxidative insults. It is, therefore, conceivable that reduction of the expression of PRDX1 and PRDX2 by nicotine and other substances of abuse would lead to ROS accumulation and add to the injury to the nervous system. On the other hand, the induction of these proteins may be a result of an increase in ROS in the cells.

4.3 Protein Modification and Degradation

Several proteins involved in modification and degradation are regulated by nicotine and other drugs. A number of chaperones are modified by nicotine treatment in rat brain (Table 13.1), including heat-shock 70-kD protein 8 (HSPA8), chaperonin 60 (HSPD1), **c**haperonin-containing t-complex polypeptide 1 subunit 6α (CCT6A), and heat-shock protein 70-kDa variant (HSC70-ps1). Moreover, HSPA8 and HSPD1 are regulated by morphine, HSPA8 by ethanol, and HSPD1 by amphetamine. Neuroproteomics analyses showed that peptidyl-prolyl isomerase A (PPIA; also known as cyclophilin A) is regulated by nicotine, morphine, and butorphanol (Hwang and Li 2006; Kim et al. 2004; Prokai et al. 2005). PPIA, an 18-kDa protein that possesses peptidyl-prolyl isomerase activity, is expressed abundantly in all tissues, including the brain (Lad et al. 1991). It can accelerate prolyl isomerization in protein substrates and thus is believed to be involved in protein folding and intracellular protein transport (Schmid 2001). Chaperones are involved in the folding of newly synthesized proteins, as well as refolding of proteins denatured under environmental stress, and thus play important roles in regulating their conformation, movement across membranes, and availability of receptors or the activity of enzymes (Sharp et al. 1999). Because of their constructive functionality, chaperones are likely to work in concert with other protein modification and degradation pathways for the plasticity and maintenance of cellular homeostasis. Thus, the regulation of these proteins may alter the balance of neuronal structure and function.

Previous studies have demonstrated that HSPs are regulated by substances of abuse. For example, HSP70, one of the major inducible heat-shock proteins in the brain, is modulated at both the mRNA and protein levels by nicotine (Canoz et al. 2006), amphetamine (Miller et al. 1991), ethanol (Calabrese et al. 2000; Canoz et al. 2006), and cocaine (Novikova et al. 2005), which is consistent with the results of proteomics study (Table 13.1). However, it is unclear whether the drug-induced changes in mRNA and protein expression of HSPs in different brain regions are secondary to a general stress-like situation after drug administration, such as oxidative stress, or a counter-regulatory action against the potentially hazardous cellular

effects of the drugs. It is likely that the toxic cellular effects of the drugs activate compensatory protective mechanisms in the neuron.

One mechanism used by the cell to sustain homeostasis under new environmental conditions is the ubiquitin–proteasome pathway. Ubiquitin is a conserved protein that targets proteins for degradation by the 26S proteasome complex. Because of the specificity of ubiquitination, ubiquitin is responsible for targeting not only denatured proteins but also proteins that are intact but regulated in a time- or region-dependent manner. Through the destruction of these key signal proteins, the ubiquitin system is an important component of many biological pathways, such as the cell cycle, signal transduction, transcriptional regulation, and endocytosis. Moreover, under stress, proteins may become denatured and nonfunctional, producing an environment that may be cytotoxic. The ubiquitin–proteasome system thus has a primary role in maintaining a healthy cellular environment by minimizing the accumulation of denatured and nonfunctional proteins.

Proteomic results showed that ubiquitin carboxy-terminal hydrolase L1 (UCHL1) is downregulated by nicotine in the NA of rats (Hwang and Li 2006). This protein also is suppressed in the PFC of human alcoholics (Alexander-Kaufman et al. 2006; Lewohl et al. 2004) and the NA of rats treated intermittently with morphine (Li et al. 2006). UCHL1 is a member of the deubiquitinating enzyme family, which can cleave polyubiquitin from target proteins and hydrolyze monoubiquitin to influence downstream signaling pathways (Nijman et al. 2005). The modification of this protein indicates that the ubiquitin–proteasome pathway is involved in the cellular response to drug-induced conditions. For example, our previous work showed that this pathway is highly regulated by chronic nicotine treatment in various brain regions (Kane et al. 2004). Proteins involved in this pathway also are differentially regulated by other drugs of abuse, such as amphetamines (Iacovelli et al. 2006), heroin, morphine (Drakenberg et al. 2006; Rambhia et al. 2005), alcohol (Donohue and Osna 2003; Gutala et al. 2004; Sokolov et al. 2003), and cocaine (Dietrich et al. 2005).

5 Concluding Remarks

By using proteomics technologies, protein expression in the nervous system in response to substances of abuse can be profiled on a large scale. Some of the proteins identified have been suggested to be associated with substances of abuse by traditional molecular technologies, but the neuroproteomics approach can provide a much broader and comprehensive view of the function and interaction of these proteins. Although at this time, it is difficult to assign a precise biological function to all the proteins identified simply on the basis of the proteomics results, determination of differentially expressed proteins and their corresponding biological pathways in response to a drug treatment should provide important insight into the mechanism underlying drug–neuron interaction. Furthermore, identification of common proteins and biological processes in response to all substances of abuse not only provides clues to the function of these proteins but also helps us to understand

the similarity between the biochemical mechanisms that neurons use to respond to different drugs of abuse.

After comparing the protein expression profiles in response to chronic nicotine exposure with those obtained by treatment with alcohol, amphetamine, butorphanol, cocaine, and morphine, we identified a few biological processes that are regulated by multiple drugs of abuse. Of them, three pathways, related to energy metabolism, oxidative stress response, and protein degradation and modification, are reviewed in the current communication. Such similarity indicates that despite the obvious differences among their chemical properties and the receptors with which they interact, all of these drugs cause similar changes in cellular activities and biological processes in the neurons. More specifically, substances of abuse may directly or indirectly disturb the structure and function of mitochondria and thus energy generation in the cell, which not only modifies the expression of energy metabolism-related proteins but also modulates other activities secondary to abnormal energy supplies. A disturbance of energy generation is accompanied by an increase in the concentration of ROS, which will change the redox status of both mitochondria and cytoplasm and consequently lead to high oxidative stress. This stress can eventually evoke a series of cellular responses such as the oxidation of protein and DNA, the induction of heat-shock proteins, and the modulation of signal transduction. Moreover, the dysfunctional mitochondria may break and cause apoptosis. Eventually, the regulation of different pathways and biological processes may lead to numerous changes in neurons such as alterations in cellular architecture, synaptic transmission, and neuronal plasticity. A detailed description of these physiological effects of multiple substances of abuse is provided in Fig. 13.1.

We have discussed only the protein profiles modulated by different substances of abuse. In order to elucidate the biological mechanisms underlying drug dependence, a reasonable approach is to compare the protein expression profiles of cells treated with substances of abuse with those of cells treated with psychotropic drugs without addiction liability. Such a systematic comparison was not performed in the current review because of its deviation from the focus of this report and the diversity of psychotropic drugs. However, based on a preliminary comparison of protein expression profiles between substances of abuse and several antipsychotic drugs such as risperidone (O'Brien et al. 2006), fluoxetine (Carboni et al. 2006; Cecconi et al. 2007; Khawaja et al. 2004), and clozapine (La et al. 2006; Paulson et al. 2007), we found that only a few proteins are regulated by both types of drugs. This indicates that different mechanisms are involved in the CNS response to these two categories of drugs. On the other hand, we found that several proteins involved in the biological processes modulated by addictive drugs also are regulated by antipsychotic drugs. For example, ALDOC and ENO1 are regulated by chronic risperidone (O'Brien et al. 2006) or fluoxetine (Cecconi et al. 2007; Khawaja et al. 2004) treatment in rats, and some subunits of ATP synthase (O'Brien et al. 2006) are modified by clozapine (La et al. 2006) or fluoxetine (Carboni et al. 2006), implying the existence of some similarities between the molecular mechanisms underlying the neuronal adaptation to the environment invoked by both antipsychotic and addictive drugs. Furthermore, the disturbance of energy metabolism by antipsychotic drugs

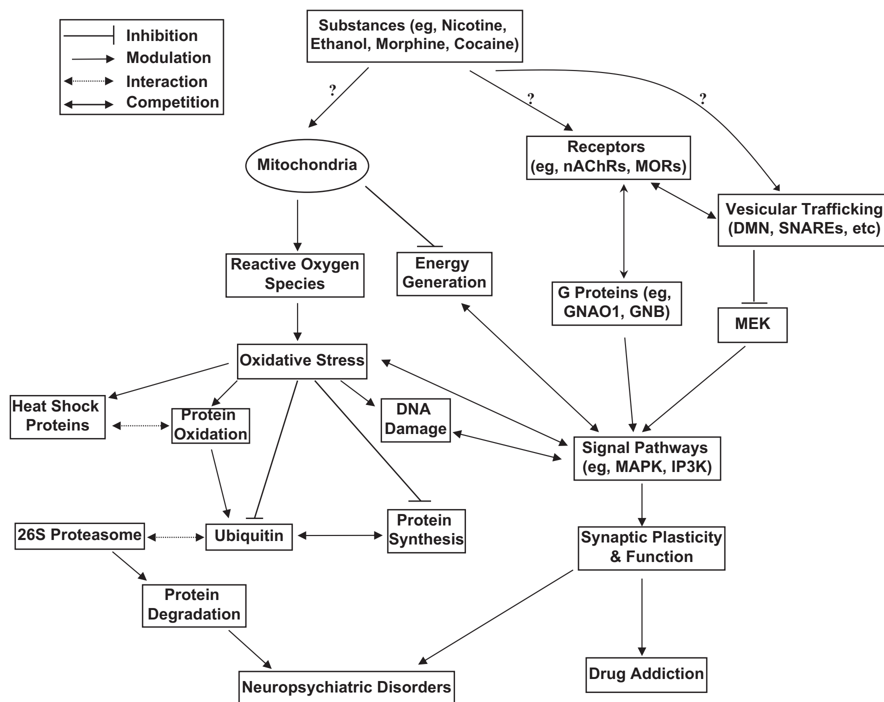

Fig. 13.1 Schematic model for the biological effects of substances of abuse on neurons. These substances modulate the structure and function of mitochondria, disturb energy generation, and produce oxidative stress. The abnormal energy supply may evoke an unfavorable intracellular environment, forcing the cell to adapt, which can affect almost every aspect of neuron activity. The oxidative stress may cause protein oxidation and DNA damage. This effect may modulate the systems responsible for maintaining homeostasis within the cell such as the ubiquitin–proteasomal system involved in protein degradation, the chaperonin system that aids in protein folding, and the antioxidant system that eliminates the ROS and RNS. The final result may be the regulation of that vesicular trafficking system closely related to synaptic transmission and modulation of signaling pathways related to cell fate and neuron plasticity. The regulation of these pathways may evoke changes in neuron viability and structure and, ultimately, drug dependence and other neuronal disease. This model is modified and expanded from our previous proposal (Kane et al. 2004; Konu et al. 2004). *Abbreviations*: *GNAO1* guanine nucleotide-binding protein, α-activating activity polypeptide o, *GNB* guanine nucleotide-binding protein β, *MAPK* mitogen-activated protein kinase, *MEK* MAPK/ERK kinase, *MOR* μ opioid receptor, *DMN* dynamin, *nAChR* nicotinic acetylcholine receptor, *PI3K* phosphoinositide-3-kinase, catalytic, α polypeptide, *SNAREs* soluble N-ethylmaleimide-sensitive factor attachment protein receptors

has been connected with a reduction in synaptic density (O'Brien et al. 2006), impairment of neuroprotection (O'Brien et al. 2006), and vesicular trafficking (Carboni et al. 2006), which may eventually lead to changes in neural plasticity. As more and more proteomic data become available, a comprehensive comparison of the protein expression patterns may help us to understand the neuronal–drug interaction mechanisms shared by addictive substances and psychotropic drugs without addiction liability, as well as those drugs specific to each category.

Much of our knowledge of drug–neuron interactions has been accumulated through relatively traditional approaches that focus on the function or interaction of one or a few genes/proteins. Such approaches are critical in our exploration of the mechanisms underlying drug addiction. However, the newly developed high-throughput protein profiling technologies such as neuroproteomics can provide information regarding protein function and interaction on a much larger scale. In addition to its ability to obtain a relatively global view of metabolism and cell signaling, the neuroproteomics approach does not require selecting target proteins in relation to the neural actions of addictive drugs. Thus, the results should be more objective, which is critical for its promise of identifying novel proteins involved in the neural effects of addictive drugs. Even in its early stages, neuroproteomics has proved to be a powerful tool for examining and identifying the dynamics of protein changes and the biological processes they are involved in that underlying drug dependence. Eventually, insights from neuroproteomics coupled with other conventional molecular technologies will not only help us to elucidate the mechanisms used by neurons with respect to their architecture, synaptic transmission, and signaling cascades in response to treatment by drugs of abuse but also can lead to the accelerated identification of new targets for the treatment of drug dependence.

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Chapter 14
Regulatory Roles of MicroRNAs in Addictions and Other Psychiatric Diseases

Abstract A central question in addiction is how drug-induced changes in synaptic signaling are converted into long-term neuroadaptations. Emerging evidence reveals that microRNAs (miRNAs) play a distinct role in this process through rapid response to cellular signals and dynamic regulation of local mRNA transcripts. Because each miRNA can target hundreds of mRNAs, relative changes in the expression of miRNAs can significantly affect cellular responsiveness, synaptic plasticity, and transcriptional events. These diverse consequences of miRNA action occur through coordination with genes implicated in addictions, the most compelling of these being the neurotrophin *BDNF*, the transcription factor cAMP response element-binding protein (*CREB*), and the DNA-binding methyl CpG-binding protein 2 (*MeCP2*). In this chapter, we summarize the recent progress in the understanding of miRNAs in general mechanisms of plasticity and neuroadaptation and then focus on specific examples of miRNA regulation in the context of addiction. We conclude that miRNA-mediated gene regulation is a conserved means of converting environmental signals into neuronal response, which holds significant implications for addiction and other psychiatric diseases.

Keywords MicroRNA · Addiction · Pathways · cAMP response element-binding protein (CREB) · DNA-binding methyl CpG-binding protein 2 (MeCP2) · Plasticity · Neurodegeneration · 3-UTR · miR-124 · *BDNF* · Memory · Nicotine dependence · Alcoholism · Cocaine dependence

1 Introduction

There is no doubt that identification of candidate genes for addiction to different substances is an essential step in elucidating the genetic mechanisms underlying addictions. It is equally important to determine what causes an observed association of a candidate gene with an addictive phenotype, i.e., what molecular mechanisms are involved. Generally speaking, we are interested in characterizing two types of polymorphisms, located in either the coding or the regulatory region of a candidate gene of interest. To some extent, it is relatively easy to understand how a non-synonymous variant impacts the function(s) of a protein. Nonetheless, it is equally

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important to concentrate on those variants located in the regulatory regions of both the 5′ and 3′ ends of a gene, as they are more common and may alter regulation of mRNA expression through changing the binding affinities of transcriptional factors or microRNAs (miRNAs).

The past decade has seen a significant shift in the conceptualization of the genome. Noncoding elements, once considered "junk sequences" (i.e., evolutionary relics), are now considered to be important regulators of gene expression that are necessary for the development and organization of complex life (Perkins et al. 2005). This new framework allows extensive novel investigation into noncoding RNA (ncRNA), as 98% of the human genome is nonprotein coding sequences (Mattick 2001).

miRNA, a relatively newly discovered class of small noncoding single-stranded RNAs of about 21–23 nucleotides and evolutionarily conserved regulatory molecules that directly target the 3′-untranslated region (3′-UTR) of mRNAs, has been implicated in modulating posttranscriptional expression of more than one-third of human genes by either enhancing or suppressing mRNA translation (Bartel 2004). MicroRNAs are endogenous to mammalian cells and are essential controllers of cellular proliferation, differentiation, and apoptosis (Chen et al. 2004; Hwang and Mendell 2006). In humans, more than 1000 miRNA sequences have thus far been identified, and more than a third of all genes are subject to miRNA regulation, with each miRNA family targeting an average of approximately 500 RNA transcripts (Friedman et al. 2009).

Several properties of miRNA regulation and processing make them ideally suited for rapid environmental response. First, their small size and noncoding nature allows them to be transcribed more quickly than other immediate–early response genes, which are much longer and must undergo the additional step of translation (Hobert 2008). Second, because miRNAs target mRNAs directly, they regulate protein synthesis at the ribosome. Furthermore, their association with the ribosome allows subcellular localization, including to dendrites (Ashraf et al. 2006; Hobert 2008). The localization of miRNAs and their processing machinery to dendritic compartments provides a means for altered gene regulation in direct response to synaptic activity, fulfilling a unique requirement of neurons for synapse-specific adaptation as distinct from cell-wide changes in gene expression (Martin and Zukin 2006).

Given their ubiquitous nature, as well as the enrichment of many miRNAs in the brain (Lugli et al. 2008; Sempere et al. 2004), it is not surprising that they have been implicated in an ever-increasing number of neurologic diseases. Involvement of miRNA has been associated with various CNS disorders such as Tourette's syndrome (Abelson et al. 2005) and Rett syndrome (Urdinguio et al. 2010); neurodegenerative diseases such as Parkinson's (Kim et al. 2007), Huntington's (Maes et al. 2009), and Alzheimer's (Bicker and Schratt 2008; Sethi and Lukiw 2009); and psychiatric disorders such as schizophrenia (Perkins et al. 2007; Zhu et al. 2009) and addictions (Huang and Li 2009a, b).

An important role for miRNAs in addiction is supported by their established role in synaptic plasticity. Long-term facilitation (LTF), wherein neuronal synapses alter in strength according to activity at the synapse, is regarded as the underlying mecha-

nism of addiction, compulsion, and dependence (Hyman and Malenka 2001; Hyman et al. 2006; Koob 2005); and drugs of abuse alter synaptic signaling in various brain regions, particularly the ventral tegmental area (VTA) (Kauer 2004), striatum (Gerdeman et al. 2003), nucleus accumbens (NAcc) (Russo et al. 2010), and prefrontal cortex (PFC) (Kalivas et al. 2005). MicroRNAs, by their modes of expression and action, thus are uniquely equipped to respond to altered synaptic signaling and create neuroadaptation.

2 miRNAs in Synaptic Plasticity and Neuronal Regulation

The extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) family is a class of signal-transducing enzymes activated by cell-surface receptors and chemical or physical stresses (Chang and Karin 2001). MAPK/ERK signaling regulates local miRNA expression via phosphorylation of the miRNA-generating complex (Paroo et al. 2009), providing a common means through which an extracellular signal can be converted rapidly to a miRNA-mediated response. There are two general types of adaptive responses under miRNA regulatory control: direct regulation of protein synthesis that plays a crucial role in plasticity at the synapse (Pietrzykowski 2010; Smalheiser and Lugli 2009) and interactions with transcription factors that appear to modulate more enduring neuroplastic changes in the entire cell.

2.1 Dendritic Morphology

Dendritic miRNAs may underlie or enhance the observed effects of key molecules in synaptic plasticity. Brain-derived neurotrophic factor (BDNF) is a neurotrophin crucial to cortical survival and maintenance, as well as the growth of new neurons and synapses (Acheson et al. 1995; Huang and Reichardt 2001). The protein can induce transcription of miRNA-containing gene loci (Remenyi et al. 2010) and interact directly with mature miRNAs (Schratt et al. 2006). Treatment of neonatal rat cortical cells with BDNF upregulates the miRNA precursor premiR-132 through its action on cAMP response element-binding protein (CREB) (Vo et al. 2005). In turn, mature miR-132 stimulates neurite outgrowth (the process preceding synapse formation) through inhibition of p250GAP, a protein that represses neurogenesis (Vo et al. 2005). Increased thickness of dendritic spines from transgenic miR-132 has been confirmed by an *in vivo* study (Hansen et al. 2010). Another miRNA-mediated role for BDNF has been found in postnatal rat hippocampal cells: the brain-specific miR-134, located in the synaptodendritic compartment, inhibits translation of co-localized Lim domain-containing protein kinase 1 (*Limk1*) (Schratt et al. 2006). Limk1 is a regulator of actin filament dynamics, necessary for dendritic spine development (Endo et al. 2003). Treatment with BDNF releases miR-134

inhibition of Limk1, thereby stimulating growth of the dendritic spine. This mechanism is reversible, with the activity state of the synapse switching translational inhibition on or off (Schratt et al. 2006), indicating a dynamic role for this miRNA in synapse plasticity.

Two recent studies provide additional evidence of localized miRNAs affecting dendritic structure (Edbauer et al. 2010; Siegel et al. 2009). In the first, miR-138 restricted dendritic growth in rat hippocampal neurons through inhibition of acyl protein thioesterase 1 (Siegel et al. 2009). Calcium influx decreased the amount of pre-miR-138 and its cleavage activity, facilitating dendritic strengthening in response to synaptic stimulation. In the second study, focusing on fragile-X mental retardation protein (FMRP)-related miRNAs in mouse hippocampal neurons, overexpression of either miR-132 or miR-125b led to opposing dendrite morphologies, with miR-132 corresponding to thicker spines and miR-125b to thinner (Edbauer et al. 2010). Interestingly, knockout of FMRP prevented these effects despite the lack of a miRNA recognition site, indicating an indirect association that regulates downstream protein targets in tandem (Edbauer et al. 2010). Taken together, these findings provide evidence that localization of miRNAs to synapses and their subsequent regulation of protein synthesis in response to specific synaptic stimuli is a significant mechanism underlying plasticity.

2.2 Gene Regulation and Memory

miRNA targeting of transcription factors such as CREB provides an additional layer of regulation with more widespread and enduring neuronal consequences. CREB-induced transcription is an important component of a switch from short-term to long-term plasticity (Barco et al. 2002), and proper CREB functioning is necessary for long-term memory formation (Benito and Barco 2010). Heightened CREB concentrations increase neuronal excitability in the amygdala and NAcc (Dong et al. 2006; Zhou et al. 2009), and the degree of CREB phosphorylation is associated with sensitization to cocaine (Marin et al. 2009) and morphine (Moron et al. 2010).

Comparative sequence analysis revealed that miRNAs expressed in neurons are highly enriched with cAMP response elements and neuron-restrictive silencing elements, implicating CREB as a positive regulator and RE1-silencing transcription factor (REST) as a negative regulator of these genes (Wu and Xie 2006). Because some neuronal miRNAs also target CREB and REST, and because all three types of regulators share neural gene targets, it is proposed that a network among CREB, REST, and miRNAs carries out coordinated gene regulation through extensive feedback. Modeled gene networks indicate that feedback circuits increase the stability and robustness of the system (Becskei and Serrano 2000), and mutual binding sites and targets shared by miRNAs and transcription factors could be seen as enabling cross talk between the genome, transcriptome, and proteome.

The most abundant miRNA in the brain, miR-124 (Lagos-Quintana et al. 2002), shows mutual targeting with REST. This antagonistic relation is important to cel-

lular differentiation and identity through opposing effects on neural and nonneural transcripts (Visvanathan et al. 2007), with miR-124 promoting a neuronal phenotype (Conaco et al. 2006). In mature neurons, miR-124 inhibits CREB in an activity-dependent manner (Rajasethupathy et al. 2009). It also responds to the neurotransmitter serotonin (5-HT) in *Aplysia* neurons with a rapid decrease in expression, leading to an increase in CREB expression and inducing LTF (Rajasethupathy et al. 2009). Under normal conditions, this effect requires five spaced pulses of 5-HT; however, when miR-124 is downregulated or CREB is upregulated, LTF can be induced after a single pulse. Thus, miR-124 and CREB can be seen to work in conjunction to mediate neural responsiveness to serotonin-induced learning.

Gao and colleagues identified another miRNA–CREB pathway important in memory (Gao et al. 2010). Deficiency of SIRT1 was found to impair plasticity and memory formation in mice and cause overexpression of miR-134, a miRNA previously implicated in dendritic morphology. miR-134 was predicted to have three binding sites in the 3′-UTR of CREB mRNA, and luciferase reporter assays confirmed direct binding. SIRT1 normally forms an inhibitory complex upstream of the miR-134 gene to regulate its expression negatively. When disinhibited, overexpression of miR-134 downregulates CREB and BDNF (which is CREB-activated) and leads to impaired plasticity and to memory deficits. Blocking miR-134 in SIRT1-knockout mice reverses these deficits (Gao et al. 2010). Intriguingly, Renthal et al. reported that chronic, but not acute, cocaine exposure increases the expression of SIRT1 in the NAcc and that SIRT1 inhibition decreases the rewarding effects of the drug (Renthal et al. 2009). Cocaine's upregulation of SIRT1 could exert the observed effects via the miRNA–CREB pathway.

Chromatin remodeling may be an additional component of a coordinated mechanism with miRNAs and transcription factors through which signaling-induced neuroadaptations gain long-term stability. miR-132, the CREB-activated miRNA involved in dendrite morphogenesis, orchestrates chromatin remodeling through regulation of MeCP2, p300, and JARID1A in the suprachiasmatic nucleus, with the effect of attenuated resetting of the circadian clock in response to light (Alvarez-Saavedra et al. 2011). MeCP2 is a DNA-binding protein that can compact chromatin structure (Georgel et al. 2003), repress transcription by competitive binding at promoters or through complex formation with histone deacetylases or corepressors (Jones et al. 1998), or activate transcription through association with CREB1 (Chahrour et al. 2008). MeCP2 is abundantly expressed in neurons and is critical to proper functioning; its overexpression and underexpression both result in detrimental neural effects, and mutations in MeCP2 underlie Rett syndrome (Martinowich et al. 2003), a disorder of the gray matter that almost exclusively affects females. There is evidence that MeCP2 regulates a cohort of microRNAs (including miR-132) through binding at promoter regions of miRNA transcription units, where it acts primarily as a repressor (Wu et al. 2010). Several of these miRNAs are synaptically enriched, and many are predicted to target BDNF, which is downregulated in the MeCP2-knockout mouse and rescues Rett syndrome-like deficits (Larimore et al. 2009). In turn, miR-132 represses MeCP2 but is activated

by BDNF, highlighting a miRNA autoregulatory loop (Wu et al. 2010), which apparently stabilizes activity-dependent BDNF production, as well as MeCP2 expression (Klein et al. 2007).

miR-132, CREB, MeCP2, and BDNF, all are important components of learning and memory (Benito and Barco 2010; Caccamo et al. 2010; Hansen et al. 2010; Lonetti et al. 2010). An emerging picture is that these distinct molecular entities form a multilevel network that responds to neural activity at the immediate level of protein functioning at the synapse but also, with continuing feedback at the transcriptional and posttranscriptional levels, carries out longer-term changes necessary for memory formation. The precise relations of such complex epigenetic networks have yet to be elucidated – particularly at the level of the miRNA, where the vast number of potential targets presents a practical challenge. However, miRNAs seem to occupy a unique position between synaptic signaling and neuronal gene expression, which holds significant consequences for memory as well as addiction.

3 MicroRNAs and Their Involvement in Addictions

As drug addictions are widely regarded as disorders of plasticity, according to reward-based learning (Hyman et al. 2006), it would be expected that miRNA-mediated mechanisms of synaptic plasticity such as those just described in functional systems contribute to formation of the addictive phenotype. During the past few years, evidence has begun to accumulate that miRNA responses to drug-induced stimuli play important roles in neuroadaptive pathways that are induced by, or react against, consistent drug exposure.

3.1 Cocaine Addiction

A study by Hollander and colleagues (Hollander et al. 2010) reported altered miRNA expression in the striatum, a brain region involved in drug-seeking habits (Belin and Everitt 2008). Increased expression of miR-132 and the closely related miR-212 were observed in rats having extended access (6 h/day) but not in rats under restricted access or "yoked" rats, who received cocaine in a response-independent manner. Increasing or decreasing miR-212 expression decreased and increased cocaine self-administration, respectively, during unlimited access, indicating that this miRNA decreases cocaine's motivational properties and protects against overconsumption. miR-212 appears to exert this effect at least in part by upregulating striatal CREB (Hollander et al. 2010). Upregulation of the cAMP pathway is a compensatory response to chronic drug exposure (Nestler and Aghajanian 1997), and elevation of CREB in the NAcc decreases the rewarding effects of cocaine (Carlezon et al. 1998). Follow-up work from the same research group (Im et al. 2010) demonstrated that MeCP2 has a homeostatic interaction with

miR-212 to control BDNF expression and cocaine intake. MeCP2 attenuates cocaine's upregulation of miR-212 and subsequent CREB signaling, whereas miR-212 inhibits MeCP2 expression. Although MeCP2 itself may act as a transcriptional repressor of BDNF in the absence of neuronal activity (Martinowich et al. 2003), concentrations of the protein coordinate closely with the amount of BDNF in the brain (Chang et al. 2006); and phosphorylation of MeCP2 regulates activity-dependent expression of BDNF (Zhou et al. 2006). Together, these observations indicate that a BDNF–MeCP2-inclusive network such as that described previously is necessarily co-expressed in response to neural activity and is engaged by cocaine. BDNF expression in the NAcc produces robust behavioral consequences, facilitating compulsive cocaine-taking behavior and increasing measured cocaine reward (Horger et al. 1999; Schoenbaum et al. 2007). As CREB induces expression of both BDNF (Choi et al. 2006) and MeCP2 (Klein et al. 2007), it seems that miR-212, by suppressing MeCP2 (and subsequently BDNF), serves as a "filter" for CREB-responsive genes (Im et al. 2010).

The upregulation of miR-212 in the striatum may reflect a mechanism of tolerance within a neuron. Each use of cocaine upregulates BDNF (Le Foll et al. 2005), and BDNF action on its TrkB receptor is one of several types of synaptic activity that induces transcription of miR-212 and miR-132 (Remenyi et al. 2010), which accounts for the observed increase in miR-212 in rats having extended access to cocaine. A sustained increase would reduce normative activity-dependent BDNF expression, which would decrease the rewarding effects of each cocaine exposure. More cocaine therefore would be necessary to achieve the same effect (Fig. 14.1).

Chandrasekar and Dreyer (2009) applied miRNA prediction software to identify miRNAs that might target cocaine-responsive genes implicated in addiction and found strong predictions for miR-124, let-7d, and miR-181a. A miRNA quantification of rat mesolimbic brain slices showed that miR-124 and let-7d were significantly downregulated, and miR-181a was significantly upregulated by chronic cocaine exposure. Further investigation (Chandrasekar and Dreyer 2011) revealed that overexpression of miR-124 and let-7d in the NAcc attenuates cocaine-induced conditioned place preference (CPP), whereas miR-181a overexpression enhances cocaine-induced CPP, and silencing of these miRNAs produces inverse effects. This study further demonstrated an impressive array of addiction-related gene expression changes under these various conditions. Notably, miR-124 and let-7d overexpression upregulated the dopamine transporter (DAT), whereas miR-181a overexpression downregulated it. Because DAT is cocaine's directly inhibited target and the source of its effects on the dopaminergic system (Luscher and Malenka 2011), these findings likely relate strongly to the observed effects of manipulation of these miRNAs on CPP, an indirect measure of cocaine reward, and reflect compensatory changes in the cases of miR-124 and let-7d and a sensitizing change in the case of miR-181a. The expression of a number of other genes is modulated by these miRNAs, including Δ*Fos* and *Fos B*, *DRD2* and *DRD3*, *Nac1*, *Per2*, *GRIA2*, and *7MYT1*, highlighting the diverse effects of miRNA dysregulation on synaptic signaling (i.e., receptors) and transcription factors.

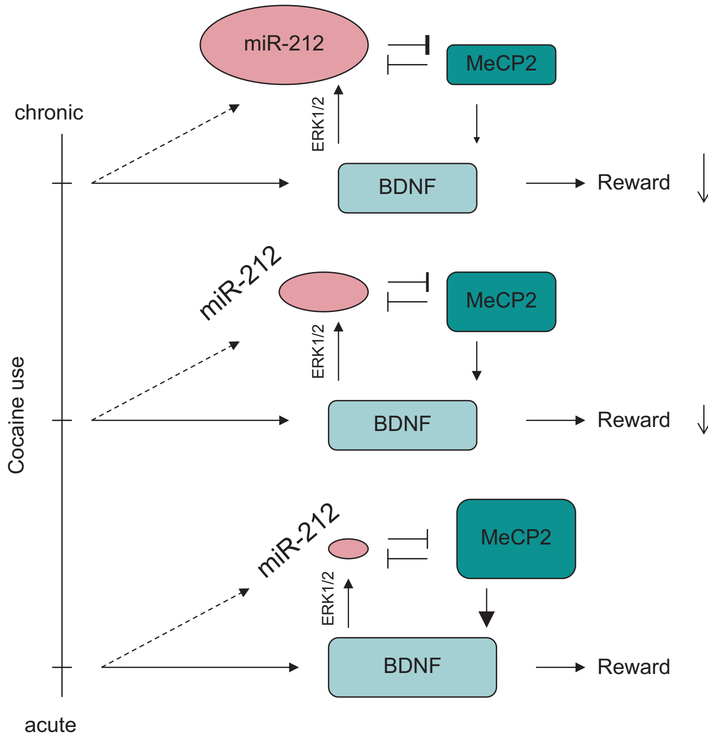

Fig. 14.1 Relation between miR-212, methyl CpG-binding protein (MeCP2), and brain-derived neurotrophic factor (BDNF) mediates the adaptive response to chronic cocaine exposure. Cocaine increases BDNF concentrations, even after a single dose (Le Foll et al. 2005), and BDNF has a strong role in the motivating and rewarding aspects of the drug (Graham et al. 2007). BDNF signaling at the synapse increases transcription of miR-212 via an extracellular signal-related kinase (ERK1/2) pathway (Remenyi et al. 2010). This miRNA exhibits mutual inhibition with MeCP2, a transcription factor necessary for BDNF expression in response to neural activity (Zhou et al. 2006). The greater expression of miR-212 observed after chronic cocaine treatment (Im et al. 2010) therefore represents a mechanism of tolerance by inhibiting activity-dependent BDNF transcription in the NAcc

In light of the networks examined in this report, the effects of these miRNA manipulations on BDNF, CREB, and MeCP2 are of particular interest. BDNF expression decreases when miR-124 is silenced, or when let-7d is either overexpressed or silenced; MeCP2 is significantly downregulated when any of the three miRNAs is silenced, with the strongest effect (a tenfold decrease) in the case of miR-124. Amounts of CREB protein also increase significantly when miR-124 is silenced (Luscher and Malenka 2011).

Although the transcriptional repressor REST was not examined in the more recent investigation, the initial study by Chandrasekar and Dreyer (2009) found that chronic cocaine exposure induces REST expression. The previously discussed antagonistic relation between miR-124 and REST might reasonably imply that this

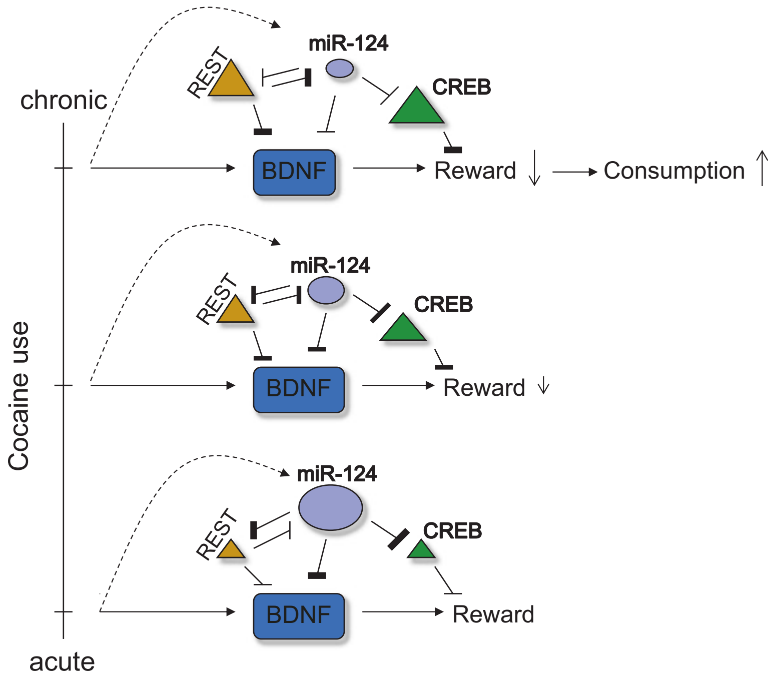

Fig. 14.2 Decreased miR-124 expression in chronic cocaine conditions allows sustained increases in cAMP response element-binding protein (CREB). miR-124 is important in learning and memory through its inhibition of CREB (Rajasethupathy et al. 2009). Following chronic cocaine exposure, this miRNA is downregulated, whereas two of its targets, CREB and RE1-silencing transcription factor (REST), are upregulated (Chandrasekar and Dreyer 2009). Both miR-124 and REST inhibit BDNF expression, so it seems that downregulation of this miRNA marks a shift in the control of BDNF inhibition from miR-124 to REST. This shift allows higher concentrations of CREB, which decreases the rewarding effects of cocaine (McClung and Nestler 2003)

is another instance of a homeostatic relation between a miRNA and a transcription factor as an adaptation to chronic cocaine exposure. miR-124 and REST both target and suppress BDNF, so it seems that REST induction might serve as a transfer control of BDNF inhibition from the translational level (via miRNA regulation) to the transcriptional level (via REST inhibition). This shift may be necessary to allow the adaptive increase in CREB that is observed in the miR-124 silencing condition, as CREB is targeted by miR-124 but not REST (Fig. 14.2). Evidence for an inverse transfer of BDNF regulatory control has been observed in the PFC, wherein BDNF mRNA correlates more strongly with mechanisms of transcriptional control (e.g., open-chromatin-associated histone H3 methylation) on BDNF gene promoters during childhood, whereas miRNAs become more prominent regulators in adolescence and adulthood (Mellios et al. 2008). As miR-124 promotes the neuronal phenotype whereas REST opposes it, the observed effects of cocaine on these factors might represent a regression into a more “immature” neuronal phenotype. The chromatin remodeling of the BDNF gene observed in response to chronic but not acute cocaine

treatment (Kumar et al. 2005; Sadri-Vakili et al. 2010) also supports such a shift in control. Regardless, the combined effects on CREB are likely relevant to learning-related aspects of addiction, such as cocaine-induced cues (Chandrasekar and Dreyer 2011), as well as general cocaine reward (Carlezon et al. 1998).

Whereas greater amounts of CREB protein in the NAcc decrease cocaine self-administration and relapse (Self et al. 1998), more BDNF in this region increases self-administration and relapse (Graham et al. 2007). This is a somewhat surprising disparity, given that CREB induces BDNF transcription but appears to be indicative of a general counterbalance between adaptations that accentuate cocaine's signaling effects and those that offset these effects (even within the same network). miRNAs such as miR-124 and miR-212 appear to mediate this balance by selective targeting and activity-dependent expression.

3.2 Nicotine Dependence

A characteristic shared feature of addictive drugs is the unconditional increase in synaptic dopamine they induce (Hnasko et al. 2005), so dopamine receptor expression represents a potentially important factor in the drug response. We recently investigated the differential expression of the dopamine receptor D1 gene (*DRD1*) in response to nicotine (Huang and Li 2009a). This gene was previously found in a genetics association study to contain a SNP rs686 significantly associated with nicotine dependence (ND) (Huang et al. 2008). Because the polymorphism rs686 was in the 3′-UTR, we hypothesized that such a significant genetic association of the polymorphism with ND might be mediated by miRNA. Investigation of candidate miRNAs revealed that mir-504 directly targeted *DRD1*, with the surprising effect of upregulating expression. Moreover, upregulation was significantly greater with the "A" allele associated with ND. This observed effect agrees with the stronger predicted binding energy of miR-504 to the transcript containing this allele, as was confirmed by assay with a miR-504 inhibitor (Huang and Li 2009a). The role of dopamine signaling in reward and motivation suggests that this miRNA-mediated pathway underlies continued smoking by increasing dopamine D1 receptor synthesis at nicotine-affected synapses. It may also affect plasticity downstream, as D1-receptor signaling phosphorylates CREB (Xing et al. 2010), and phosphorylated CREB in the NAcc is necessary for nicotine-induced conditioned place preference (Brunzell et al. 2009).

In another study, we used a miRNA microarray approach to investigate the broad effects of nicotine stimulation on miRNA expression in rat PC12 cells (Huang and Li 2009b). From several hundred probe sets, 25 miRNAs were found to show significant changes, evidence that nicotine exerts specific but widespread effects on miRNA regulation. One of these, miR-140*, showed a strong predicted binding site on dynamin 1 (Dnm1), a large GTPase important for synaptic endocytosis that is significantly associated with ND (Xu et al. 2009). We subsequently demonstrated that this miRNA is greatly upregulated in response to nicotine treat-

ment and binds directly to Dnm1 to inhibit its expression (Huang and Li 2009b). Moreover, dynamin 1 was revealed in a protein interaction analysis to bind to the β2 subunit of nAChRs, a crucial component of the most abundant and highest-affinity α4β2*-containing nAChR (Kabbani et al. 2007). Dynamin 1 may play a key role in chemical dependence through its action in signal termination of G-protein-coupled receptors (GPCRs), which include dopamine and opioid receptors; and changes in the sensitivity of these receptors underlie acute drug effects (Koob and Nestler 1997). Moreover, morphine has been distinguished from nonaddictive analogs by exhibiting a deficient ability to induce endocytosis of its receptor, which disrupts signal termination and desensitization (Whistler et al. 1999). As dynamin 1 is crucial to endocytosis of GPCRs (Artalejo et al. 2002), its downregulation by miR-140* might contribute to the highly addictive aspects of nicotine, such as tolerance and craving.

Interestingly, several of the miRNAs found in this study to undergo nicotine-induced changes in expression also have been implicated in schizophrenia and other neurodegenerative disorders. For example, miR-181b, which is upregulated by nicotine, is upregulated in the temporal cortex of schizophrenic patients (Beveridge et al. 2008), whereas miR-30a-5p (one of the miRNAs that target BDNF in the PFC) (Mellios et al. 2008) and miR-29c are both downregulated by nicotine and are downregulated in the PFC of postmortem brains in schizophrenia (Perkins et al. 2007). As schizophrenia and ND show a strikingly high degree of comorbidity (Volkow 2009; Williams et al. 2010), in that most schizophrenic persons smoke, a more directed study of shared miRNA mechanisms in the two disorders could be telling; it is possible that miRNAs account for the comorbidity, either through exacerbation of psychotic symptoms in response to drug use or by exerting similar effects in response to both antipsychotics and cigarettes, thus fitting with a self-medication hypothesis.

miRNAs also appear to link nicotine with Alzheimer's disease. miR-125b was found to be upregulated by nicotine in the hippocampus of AD patients (Lukiw 2007), whereas miR-93, upregulated by nicotine, is downregulated in the cortex in AD (Hebert et al. 2008). Perhaps the most compelling correlate is miR-328, which is upregulated by nicotine and appears to play a significant role in the etiology of AD. Studies using postmortem tissues have revealed higher concentrations of β-amyloid precursor protein-converting enzyme protein (BACE1) in the brains of AD patients (Fukumoto et al. 2002; Holsinger et al. 2002), which leads to the buildup of β-amyloid, a major component of the senile plaques etiologic of AD and thought to be responsible for neurodegeneration (Kihara et al. 1997). BACE1 is a predicted target of miR-328, and in a rodent model of AD, this miRNA targeted and suppressed BACE1 expression (Boissonneault et al. 2009). Because nicotinic receptor stimulation protects neurons against β-amyloid toxicity (Kihara et al. 1997), it is tempting to speculate that miR-328 upregulation by nicotine is a component of the pathway underlying this protective effect. Regardless, the appearance of nicotine-responsive miRNAs in the etiology of neuropsychiatric disorders generally supports a role for these regulators in neural functioning. Table 14.1 provides a list of miRNAs dysregulated both in response to drugs and in neuropsychiatric illness.

Table 14.1 miRNAs responsive to drugs of abuse which have also been associated with neuropsychiatric disorders

miRNA	Targeted gene(s)	Biological function(s)	Associated diseases
mir-124	REST Visvanathan et al. (2007)	Neuronal identity (Visvanathan et al. 2007)	Cocaine addiction (Chandrasekar and Dreyer 2009), Alzheimer's disease (Maes et al. 2009)
	CREB Rajasethupathy et al. (2009)	5-HT-induced learning (Rajasethupathy et al. 2009)	
	BDNF Chandrasekar and Dreyer (2009)	Plasticity (Chandrasekar and Dreyer 2009)	
miR-132	P250GAP Vo et al. (2005)	Neurogenesis (Vo et al. 2005)	Cocaine addiction (Hollander et al. 2010), Huntington's disease (Maes et al. 2009)
miR-181b	VSNL1 Beveridge et al. (2008)	Intracellular signaling (Beveridge et al. 2008)	Nicotine dependence (Huang and Li 2009b), schizophrenia (Beveridge et al. 2008)
	GLIA1 Beveridge et al. (2008)	Neurotransmission (Beveridge et al. 2008)	
miR-30a-5p	BDNF Mellios et al. (2008)	Plasticity	
miR-29c			
miR-125b	Lin-28 Maes et al. (2009)	Neurogenesis (Maes et al. 2009)	Nicotine dependence (Huang and Li 2009b), Alzheimer's disease (Maes et al. 2009)
	NR2A Edbauer et al. (2010)	Neurotransmission/plasticity (Edbauer et al. 2010)	
miR-93	VEGF Long et al. (2010)	Cellular signaling (Long et al. 2010)	
miR-328	BACE1 Boissonneault et al. (2009)	Axon guidance, potentiation (Santarelli et al. 2011)	ND (Huang and Li 2009b), AD (Maes et al. 2009), schizophrenia (Santarelli et al. 2011)

3.3 Alcoholism

Alcohol exposure induces differential expression of about 2% of miRNAs in murine liver (Dolganiuc et al. 2009). Many of these miRNAs also are expressed in the brain, so it will be important to see whether alcohol exerts similar effects in this context (Pietrzykowski 2010). In murine striatal neurons and adult rat neurons, miR-9 undergoes significant upregulation in response to alcohol and appears to contribute to alcohol tolerance through its regulation of the BK channel (Pietrzykowski et al. 2008). This channel is highly relevant to neuronal function, as it regulates excitability, shaping of action potentials, and neurotransmitter release (Pietrzykowski 2010; Shipston 2001). In mammals, alcohol evokes tolerance in the BK channels (Martin et al. 2004). Intriguingly, miR-9 preferentially targets and degrades transcripts of BK channel isoforms sensitive to alcohol potentiation, whereas transcripts encoding alcohol-tolerant channels tend to lack miR-9 binding sites in their 3′-UTRs (Pietrzykowski 2010). Thus, alcohol-induced upregulation of miR-9 shifts BK

channel expression toward more tolerant isoforms. miR-9 also targets DRD2 (Pietrzykowski 2010), and lower expression of this receptor has been associated with alcohol abuse (Volkow et al. 2006), indicating that this miR-9 might influence the rewarding effect of alcohol in addition to its involvement in tolerance.

A systems genetic analysis of alcohol consumption has found that variations underlying GABAergic brain function contribute a significant genetic component and that G-protein subunit beta 1 (Gnb1) represents a candidate transcript for miRNA regulation relevant to alcohol consumption based on differential 3′-UTR sequences (and predicted binding affinities) between various intensities of alcohol consumption (Saba et al. 2010). The strongest target predictions across multiple software platforms were for miR-101a/b and miR-218. Subsequent studies to investigate the actual effects of these miRNAs on alcohol consumption will be necessary for confirmation. In support of a role for regulation of the GABAergic system in alcoholism, infusion of a $GABA_A$ alpha siRNA vector (pHSVsiLA2) into the central nucleus caused a reduction in binge drinking in alcohol-preferring rats (Liu et al. 2011). This study represents a promising implementation of gene therapy, given the successful behavioral effect and the tight control of the microinfusion to specific brain regions (Liu et al. 2011).

3.4 Concluding Remarks

From initial drug exposure to chemical dependence and addiction, there is a panoply of molecular changes that comprise neural adaptation. Recent studies on the effects of drugs of abuse on miRNAs reveal that these small regulatory molecules can play either a contributing role in the development of addiction, as in the case of miR-504 increasing *DRD1* expression, or a counteractive role against drug stimulatory effects, as in the case of miR-212 upregulating CREB. These converse biological effects represent the “pull” and “push” of addiction: the response of motivation-based learning networks that respond to perceived reward vs. the counterresponse of neuronal homeostasis against sustained alterations in extracellular signaling. Although these effects of sensitization and tolerance are divergent, both appear to be multilevel adaptations, spanning from short-term changes in signaling cascades to long-term changes in baseline gene expression. miRNAs can respond to synaptic signals (e.g., miR-124’s response to 5-HT) and regulate local protein synthesis but also mediate transcription factors and chromatin remodelers; thus, we propose that they are uniquely suited for neuroadaptation by converting short-term into long-term plasticity.

Plasticity relies on coordinated changes among vastly complex molecular networks, and drugs of abuse seem to exert their effect, not via a single member of the network but through coordination. However, one conserved mechanism among these gene networks appears to be miRNA-constrained feedback loops, wherein a drug-induced stimulus acts as an impetus for a change in gene expression through a temporary effect on a miRNA or transcription factor before a balance is restored

through feedback. Particularly among activity-dependent species, such as BDNF, CREB, and MeCP2, in which precise spatiotemporal regulation is essential, feedback loops would be necessary for both stability and efficiency during complex associative changes within the molecule. Furthermore, the observations of miRNA–CREB interactions in mediating neuronal responsiveness suggest a role for miRNAs as markers of recent neural activity, thus providing a context for subsequent network activation. A more precise investigation of the temporally dependent effects of neuronal (and particularly hippocampal) miRNA expression compared with early activation genes might further elucidate this question.

A fuller characterization of miRNA species and their targets will be crucial to a greater understanding of this type of gene regulation and to practical application. Deep sequencing studies are already creating large-scale profiles of miRNA populations, but it also will be necessary to characterize differential miRNA expression among various cell types and at specific synapses to fully understand their functional roles. Given the growing specificity of our knowledge of miRNA targets, their ability to modulate numerous downstream targets makes them attractive as potential therapeutic targets, because such manipulations might affect an entire network rather than a single species. In addiction especially, the potential to inhibit the longer-term adaptations to drugs of abuse would be helpful in stopping the progression of the disorder and decreasing relapse risk. Of course, the enduring risks of such gene manipulations must be addressed more thoroughly in preclinical trials than we have yet seen.

Although miRNAs appear uniquely situated to participate in cross talk between cellular signaling and long-term gene expression, the precise means and the degree of specificity of such a phenomenon are a mystery. Within a neuron, the relative extent of expression of a gene, e.g., BDNF, will certainly affect its chromatin structure, in essence to match the demand. Is it possible that the corresponding amounts of the gene's targeting miRNA, e.g., miR-132, might also be factored into the gene's chromatin remodeling? In this example, the answer seems to be yes, through miR-132's regulation of MeCP2, which in turn regulates BDNF. The tantalizing possibility, however, is that something similar is happening on a much larger scale, perhaps using miRNAs combinatorially.

Although the full power of miRNAs as gene regulators remains to be seen, they certainly seem to play a significant role in proper brain functioning. Their adaptive nature is distinctly suited for a role in addiction, but their dysregulation also is being observed increasingly in schizophrenia and Parkinson's and Alzheimer's diseases. As our ability to understand gene networks increases in both scope and precision, we will certainly want to be attentive to these tiny regulatory molecules, as the early evidence suggests they may serve as critical links.

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Chapter 15
Tobacco Smoking, Food Intake, and Weight Control

Abstract Beyond promoting smoking initiation and preventing smokers from quitting, nicotine can reduce food intake and body weight and thus is seen as desirable by some smokers, perhaps especially women. During the last several decades, the molecular mechanisms underlying the inverse correlation between smoking and body weight have been investigated extensively. This appears to be especially true for the stimulation by nicotine of α3β4 nAChRs receptors, which are located on proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC), leading to the activation of the melanocortin (MC) brain circuit that is associated with food intake. Further, α7- and α4β2-containing nAChRs have been implicated in energy homeostasis, and the effects of peripheral hormones such as leptin, ghrelin, and peptide YY (PYY) are mediated by alterations in the MC circuit. This chapter summarizes current understanding of the regulatory effects of nicotine on food intake and body weight according to the findings from the pharmacological, molecular genetics, electrophysiological, and feeding studies on these appetite-regulating molecules, such as α3β4, α7, α4β2 nAChRs, neuropeptide Y, POMC, melanocortin 4 receptor, agouti-related peptide (AgRP), leptin, ghrelin, and PYY.

Keywords Body weight · Food intake · nAChRs · α3β4 · α7 · α4β2 nAChRs · Neuropeptide Y · POMC · Melanocortin 4 receptor · Agouti-related peptide (AgRP) · Leptin · Ghrelin · PYY

1 Introduction

Tobacco smoking and obesity are the two largest causes of many complex diseases (Haslam and James 2005; Jha 2009). In the United States, almost a third of adults are considered obese (Flegal et al. 2012), and roughly 20% of the adult population smoke. The prevalence of smoking has declined by an average of 1.4% each year, whereas body mass index (BMI) has increased by an average of 0.5% per year over the 15 years prior to 2005 (Stewart et al. 2009). Some smokers, especially women, report that they smoke as a means of weight control. Such concern about the inverse correlation between smoking and body weight has made smoking cessation even harder, as

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smokers generally have a notably lower body weight than non-smokers and quitters; and thinness is lauded by current advertising (Aubin et al. 2012; Hussain et al. 2012).

Population-based epidemiology studies have confirmed the inverse correlation between smoking and body weight (Albanes et al. 1987; Klesges et al. 1998). Moreover, animal studies support the view that nicotine decreases food intake (Grunberg et al. 1987). Human studies of nicotine's effects on body weight also demonstrate the inverse association of smoking with body weight (Grunberg 1991). When balb/C mice were exposed to cigarette smoke, food intake decreased significantly beginning the first day, weight loss became evident within two days, and the brown fat and retroperitoneal white fat masses shrank significantly (Chen et al. 2005). Nicotine reduces body weight gain in normal-fat-diet mice, whereas weight gain occurs in normal-fat and high-fat diet groups, which is ascribed to a decrease in energy expenditure during nicotine withdrawal (Hur et al. 2010).

Many studies have shown that food intake and body weight are regulated by susceptibility genes for obesity, which alter appetite by binding to their specific receptors (Rankinen et al. 2006; Yang et al. 2007). By investigating these genes, researchers have identified various neurotransmitters, hormones, and genes underlying the alterations in body weight induced by smoking. These include (1) α3β4, α7, and α4β2 nAChRs; (2) orexin, neuropeptide Y (NPY), proopiomelanocortin (POMC), and melanocortin 4 receptor (MC4R), which are expressed in the brain; (3) ghrelin, peptide YY (PYY), oxyntomodulin, cholecystokinin (CCK), and glucagon-like peptide-1 (GLP-1), which are produced by the gastrointestinal tract, and adiponectin, leptin, interleukin-6, and UCP, which are synthesized by adipose tissue; and (4) small neurotransmitters such as glutamate, γ-aminobutyric acid (GABA), norepinephrine, dopamine, and serotonin.

2 Nicotine Acetylcholine Receptors and Body Weight

2.1 Overview

Nicotine suppresses appetite and food intake, which contributes to the inverse correlation between smoking and body weight. Subtypes α3β4, α7, and α4β2 nAChRs have been implicated in the regulation of body weight by nicotine (Marrero et al. 2010; Mineur et al. 2011). The α7- and β2-containing (β2*) nAChRs are the most widely distributed subtypes in the mammalian brain (Dani and Bertrand 2007; Picciotto and Mineur 2014). Radioligand receptor binding assays have revealed α7 and β2* nAChRs in the ARC (Han et al. 2000, 2003). Unlike the wide distribution of α4β2 nAChRs in the mammalian brain, the α3 and β4 subunits are distributed primarily in two major cholinergic tracts, the medial habenula and the interpeduncular nucleus (Grady et al. 2009).

These channel-receptor complexes arise from combinations of subunits and respond to special activators (agonists) or inhibitors (antagonists), such as nicotine and similar compounds (Dani and Bertrand 2007). In *in vitro* studies, these com-

pounds have been used as tools in mechanism research on nAChRs, exploring the correlation between smoking and weight change. Cytosine is a full agonist of α3β4 nAChRs but a weak partial agonist of β2* nAChRs (Luetje and Patrick 1991; Papke and Heinemann 1994). Mice treated with cytosine show a robust decrease in food intake and body weight, implicating α3β4 nAChRs as weight-regulating molecules (Mineur et al. 2011). Levamisole, an allosteric modulator of α3β4 nAChRs, suppresses weight gain in mice consuming a high-fat diet (Lewis et al. 2017). AT-1001, a partial agonist of α3β4 nAChRs in humans, causes receptor desensitization at the concentrations required for activation (Zaveri et al. 2015). Sazetidine-A (SAZ-A), a relatively selective ligand of β2* nAChRs with high affinity for α4β2 nAChRs, is a β2* nAChRs desensitizer that can reduce food intake and lower body weight in mice (Dezfuli et al. 2016). Methyllycaconitine and dihydro-β-erythroidine (DHβE) are the selective antagonists of α7 nAChRs and β2* nAChRs and can inhibit nicotine-induced activation of POMC neurons in mice (Huang et al. 2011).

2.2 α3β4 nAChRs

Mice treated with nicotine and cytosine showed dose-dependent limits on their weight gain, confirming the suppressive effect of the α3β4 nAChRs on body weight (Mineur et al. 2011). In contrast, when Mineur et al. (2011) employed an adeno-associated virus (AAV) vector to knockout (KO) β4* nAChRs in the ARC, cytosine treatment failed to reduce food intake. POMC neurons are among the best-known appetite-inhibiting cells in the mammalian brain. The observation that administration of nicotine or cytosine induces the activation of POMC neurons in the ARC, as measured by *c-fos* immunoreactivity, reveals the mechanism of cytosine action on food intake (Mineur et al. 2011). Moreover, MC4R KO in the paraventricular nucleus (PVN) blocks cytosine- and nicotine-induced hypophagia, indicating that MC4R-expressing neurons play an important role in the appetite regulation induced by activation of α3β4 nAChRs (Mineur et al. 2011).

The MC system is critical in the regulation of body weight (Schwartz et al. 2000), and in this system, MC4R activation by melanocortins contributes to the regulation of both food intake and energy expenditure (Tao 2010). Thus, the nicotine-induced activation of α3β4 nAChRs could interact with both POMC neurons and MC4R-dependent MC pathways to regulate body weight (Mineur et al. 2011). β4* nAChRs are thought be involved in the regulatory effect of nicotine on food intake, which distinguishes these receptors in the rewarding and reinforcing properties of nicotine, as β4 KO in the ARC of mice produces a resistant effect of cytosine on feeding behavior, whereas β2 KO mice do not show the same effect (Mineur et al. 2011; Picciotto et al. 1998). In addition, α4β2 and α6β2 nAChRs are implicated in some smoking behaviors, whereas α3- and β4-containing nAChRs show effects on appetite, food aversion, and withdrawal (Marks et al. 2015). Therefore, the β4 subunit of nAChRs is considered a therapeutic target for appetite control without addiction liability (Picciotto and Mineur 2013).

Human genetic association studies have demonstrated that variants in the *CHRNA5/A3/B4* gene cluster are significantly associated with the risk of smoking initiation (see Chap. 6). Genome-wide association studies (GWAS) of body mass index (BMI) and ND have revealed different variants associated with BMI and smoking-related behavior. Notably, rs1051730, the SNP most strongly associated with nicotine dependence (ND), correlates with reduced BMI in current and former smokers but does not have a significant effect on the BMI of never smokers (Freathy et al. 2011). This implies a role for variants in the *CHRNA5/A3/B4* gene cluster in the regulatory mechanism of smoking effects on body weight, with actions on both the body and the brain.

2.3 α4β2 nAChRs

Nicotine treatment increases α4β2 nAChRs in the mouse, rat, and human brain (Marks et al. 2015). nAChR ligands, including cytosine, anatoxin, and varenicline, increase the density of α4β2 nAChRs *in vivo*. However, SAZ-A, a partial agonist and desensitizer for α4β2 nAChRs, not only failed to increase the density of receptor subtypes in rodent brain but also maintained the increased nAChRs induced by chronic administration of nicotine (Hussmann et al. 2014). In addition, chronic nicotine exposure reduces the expression of α6β2 nAChRs in rodent and monkey brain (Marks et al. 2015). Both the α4 and β2 subunits have reinforcing properties relative to nicotine (Picciotto et al. 1998; Tapper et al. 2004), and the α4β2 nAChRs are associated with the rewarding effects of nicotine through mesolimbic dopamine release (Corrigall et al. 1992; Graupner et al. 2013; Maskos et al. 2005; McCallum et al. 2006).

The α4β2 receptors have high affinity for nicotinic agonists and desensitize slowly (Proulx et al. 2014). Administration of SAZ-A significantly reduces food intake and weight gain in obese mice (Dezfuli et al. 2016). In contrast to the steady and significant decrease in body weight seen in normal animals, SAZ-A treatment caused no significant weight decrease in mice when the β2 nAChR subunit was deleted. The reduced effect of SAZ-A on food intake is significantly less in β2 KO mice, indicating that β2-containing nAChRs are essential to the drug's suppressive effect on food intake and body weight (Dezfuli et al. 2016). In addition, treatment with both SAZ-A and nicotine in rats reduced weight gain over a 6-week period (Hussmann et al. 2014). Chronic nicotine treatment increased the density of α4β2-containing nAChRs in mouse and rat brain (Mao et al. 2008; Marks et al. 2011; Moretti et al. 2010), and a similar increase was observed in autopsied brains of smokers (Breese et al. 1997; Perry et al. 1999).

Thus, it is likely that food and nicotine share a central reward pathway (Chen et al. 2012). α4β2 nAChR subtypes, located in the brain reward circuits for drugs of abuse (Marks et al. 1992; Zoli et al. 2002), are found in dopamine neurons, where they regulate nicotine-induced dopamine release. Genetic studies confirm the involvement of α4 and β2 subunits on dopamine or GABA neurons in nicotine self-administration, reinforcement, and tolerance (Nashmi et al. 2007; Tapper et al. 2004).

2.4 α7 nAChRs

Distinct from most αβ heteromeric nAChRs, the α7 subunit forms mainly homomeric nAChRs in mammals (Dani and Bertrand 2007). In common with β2* nAChRs, α7 nAChRs are the most widely expressed subtypes and show similar expression patterns in rodent and primate brains (Picciotto and Mineur 2014; Proulx et al. 2014). The α7 nAChRs also are the most abundant nAChR subtypes in the hippocampus (Albuquerque et al. 2009). Expression of α7 nAChRs has been discovered in adipose tissue and macrophages in mice (Wang et al. 2011), and there is evidence of intrinsic expression of nAChRs in white adipose tissue (WAT) (Andersson and Arner 2001). However, α7 nAChRs also are widely distributed in the immune system (see Chap. 16), having a secondary role in pathogen defense (Kalkman and Feuerbach 2016). Compared with other nAChR subtypes, α7 nAChRs have a lower affinity for acetylcholine (ACH) and higher permeability for calcium (Albuquerque et al. 2009). Recent study revealed that α7 nAChRs play a key role as peripheral factors linked to body weight (Cancello et al. 2012).

α7 nAChRs are not associated only with psychiatric and neurological disorders (Proulx et al. 2014) but also are involved in the regulation of inflammatory cytokines, such as interleukin-1 (IL1), IL18, and tumor necrosis factor alpha (TNF-α) (see Chap. 16). The cholinergic anti-inflammatory pathway can be mediated by activation of the vagus nerve and α7 nAChRs, expressed by macrophages (Bencherif et al. 2011), which suppress genes in the classical inflammatory pathway. Activation of α7 nAChRs reportedly inhibits the expression of inflammatory cytokines (Wang et al. 2011), and α7 nAChR-specific antagonists block nicotine-induced cytokine inhibition (Cheng et al. 2007). α7 nAChRs agonists activate the cholinergic anti-inflammatory pathway (Cheng et al. 2007). Lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines, such as TNFα and IL-1β, is increased in α7 nAChRs KO mice (Wang et al. 2003). Such mice do not show any effect of nicotine on the suppression of pro-inflammatory cytokines (Kalkman and Feuerbach 2016). A number of studies have reported that low-grade chronic inflammation is related to severe human obesity and insulin resistance (Bouloumie et al. 2005; Bourlier and Bouloumie 2009; Cancello and Clement 2006; Weisberg et al. 2003; Xu et al. 2003).

Long-term stimulation of α7 nAChRs improves insulin sensitivity in rat adipocytes (Marrero et al. 2010), and α7 nAChR agonists reduce body weight and improve metabolic parameters (Marrero et al. 2010). Obese human subjects have reduced α7 nAChRs expression, and inflammatory gene expression is modulated by this nAChR subtype in human adipocytes (Cancello et al. 2012). Using a diabetes mouse model, an α7 nAChR-selective agonist (TC-7020) was linked to less food intake and weight gain, as well as to less expression of pro-inflammatory cytokines (Marrero et al. 2010). Moreover, the reverse effect is caused by its antagonist, MLA (Marrero et al. 2010). The effect of nicotine on anti-inflammatory cytokines (Lakhan and Kirchgessner 2011; Wang et al. 2003) may explain why smokers have a lesser prevalence of some inflammatory diseases (Lakhan and Kirchgessner 2011) as well as lower body weight. Rodents treated with nicotine or the α7 nAChRs agonist PNU-

282987 demonstrate greater insulin sensitivity, which is not observed in α7 nAChRs KO animals (Xu et al. 2012). Using genetically or dietetically obese mice, through acting on α7 receptors, nicotine exerted less action on WAT inflammation and better effects on glucose homeostasis and insulin sensitivity (Lakhan and Kirchgessner 2011; Wang et al. 2011).

3 Effects of Nicotine on Genes That Regulate Food Intake and Body Weight

Over the past 20 years, our understanding of the contribution of brain circuitry to the control of appetite and food intake has increased rapidly. The widely expressed nAChRs, especially the well-studied α3β4, α4β2, and α7 nAChR subunits, are the vital targets that mediate nicotine's effects on energy homeostasis. As an important discovery, the brain MC system appears to be a critical regulator of body weight (Schwartz et al. 2000). In peripheral tissue, the MC system regulates the color of the skin and hair. However, genetic and pharmacological data indicate that activation of the brain MC4 receptor decreases food intake and promotes weight loss (Schwartz et al. 2000). This receptor activity is mediated by a complex interaction between POMC neurons, which synthesize the precursor required to activate this receptor, and AgRP neurons, which synthesize an endogenous antagonist (Seeley and Sandoval 2011). Mineur et al. reported that activation of hypothalamic α3β4 nAChRs stimulates POMC neurons (Mineur et al. 2011). In contrast, POMC KO mice showed no significant difference in food intake in response to either nicotine or cytosine (Mineur et al. 2011).

The effects of peripheral hormones such as leptin, ghrelin, and PYY are mediated by alteration of the MC system. Both NPY and AgRP neurons express leptin, ghrelin, and PYY receptors, project onto POMC neurons, and release NPY by binding to the Y1 and Y2 receptors of NPY and AgRP by binding to MC3R, and GABA, resulting in inhibition of POMC neurons. Figure 15.1 depicts the interactions between these neurons and various molecules. In the following sections, we focus on the best-documented molecules regulated by nicotine and acting as regulators of appetite and body weight.

3.1 NPY

NPY, a 36-amino acid neuropeptide, is the most powerful and abundant orexigenic peptide in the mammalian brain, being especially abundant in the hypothalamus (Allen et al. 1983), although it is distributed in both central and peripheral neurons. Within the hypothalamus, NPY is produced primarily by the neurons in the ARC. NPY was discovered in mammalian brain tissue but is also found in

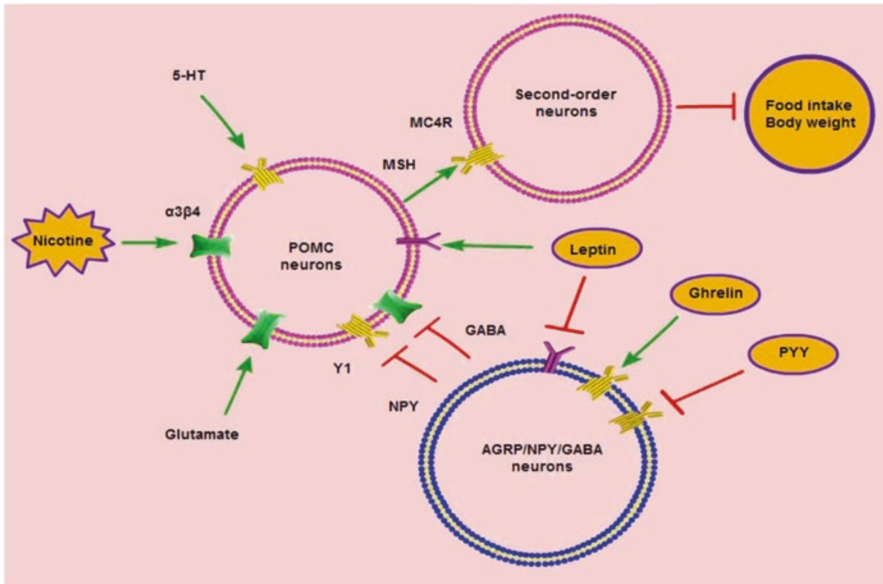

Fig. 15.1 Appetite-suppressant effects of nicotine in the brain. Appetite-regulated molecules modulate brain neuron activity through excitatory (green) and inhibitory (red) signal transduction mechanisms. The interaction of AGRP/NPY/GABA neurons and POMC neurons regulates the activity of MC4 receptors on second-order neurons involved in regulating food intake, powerfully suppressing appetite and weight gain

peripheral locations, such as the intestine and adrenal glands (Higuchi et al. 1988; Kageyama et al. 2012). The peptide can be distributed to locations without synaptic connections by passing the blood–brain barrier (Kastin and Akerstrom 1999). NPY is included in the pancreatic polypeptide (PP) family because of its 70% sequence identity with peptide YY (PYY). NPY receptors belong to the G-protein-coupled receptor superfamily, and six subtypes have been identified. Among them, the Y1, Y2, and Y5 subtypes play important roles in mediating NPY-induced feeding. NPY is a noradrenergic co-transmitter (Burnstock 1987). It also affects dopamine-related pathways (Josselyn and Beninger 1993).

NPY is linked to food intake and can regulate appetite and energy homeostasis (Chen et al. 2012; Schwartz et al. 2000). Most studies have found that exogenous NPY administration stimulates food intake and increases body weight. Food intake is related to greater NPY secretion in the PVN at the onset of darkness. In response to fasting (when leptin falls rapidly) and chronic food restriction, the expression of NPY mRNA in the ARC increases (Brady et al. 1990). In addition, treatment with exogenous leptin inhibits the overexpression of NPY (Ahima et al. 1996; Stephens et al. 1995). NPY-induced feeding behavior is inhibited by central or peripheral treatment with naloxone, an opioid antagonist (Kotz et al. 1993; Levine et al. 1990; Rudski et al. 1996; Schick et al. 1991). Both NorBIN (a KOR antagonist) and

β-FNA (an MOR antagonist) suppress NPY-induced orexigenic action, whereas naltrindole (a DOR antagonist) stimulates it (Kotz et al. 1993).

NPY neurons can be activated by nicotine. Reduced hypothalamic NPY mRNA is reported in rats after acute nicotine administration, but increased NPY mRNA accompanied by decreased food intake is seen with chronic administration (Frankish et al. 1995; Li et al. 2000a). Nicotine greatly increases NPY expression at both the mRNA and peptide levels (Li et al. 2000a). In addition, although chronic exposure to nicotine upregulates expression of NPY (Li et al. 2000a), downregulation of the expression of its receptor is observed in the rat hypothalamus (Li et al. 2000b). Reduced hypothalamic NPY receptor density in response to nicotine might explain the decrease in food intake in smokers (Kane et al. 2001). Despite the inverse correlation between nicotine exposure and feeding, NPY could be considered a stimulator of food intake, although nicotine's effects on the change in NPY expression have been inconsistent in different studies. The discrepancy could be attributable not only to differences in drug dosage but also to the method of nicotine administration. For example, Hiremagalur and Sabban (1995) observed that NPY mRNA increased in the adrenal gland only with subcutaneous nicotine injection, not if nicotine was infused by osmotic minipumps.

Smokers show less NPY expression than non-smokers, and smoking cessation results in increased NPY (Hussain et al. 2012), which might explain the weight gain common after smoking cessation from an epidemiological point of view. In this study, smokers had reduced NPY concentrations, even after adjusting for anthropometric parameters, and NPY correlated significantly with body weight, BMI, and waist circumference (Hussain et al. 2012).

NPY- and POMC-containing neurons in the hypothalamus are orexigenic and anorexigenic separately. The effect of nicotine on weight control could be attributable to the complicated interaction between neuropeptides, including NPY and POMC, in the central and peripheral nervous systems. Considering the inverse effect of nicotine on POMC and NPY neurons, at least three hypotheses have been proposed to explain how nicotine reduces body weight. First, the firing of NPY neurons induced by nicotine is not sustained by POMC neurons, which may explain why c-*fos* activation is detectable primarily in POMC, not NPY, neurons after nicotine administration (Kageyama et al. 2012). Second, apart from the excitation of NPY neurons by nicotine, inhibition of glutamate release was observed, but this effect was not seen in POMC neurons (Huang et al. 2011). Third, the nicotine-induced depolarizing effect on orexigenic NPY neurons is less than that on anorexigenic POMC neurons. In addition, nicotine withdrawal is associated with upregulated NPY and AgRP, but downregulated UCP3, in the hypothalamus (Fornari et al. 2007), which might explain the greater eating motivation and lesser energy expenditure after smoking cessation.

3.2 POMC

The MC system is one of the most important pathways associated with appetite and energy homeostasis, and about 4% of genetic obesity is explained by mutations in this system (Horvath et al. 2004). POMC is the precursor of the melanocortin protein family, including adrenocorticotrophic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin, and acts by binding to specific receptors (MCRs) (Schwartz et al. 2000). Activation of POMC cells in the ARC is associated with less food intake and more energy expenditure (Williams and Schwartz 2005), and dysfunction of POMC is related to obesity in humans and animals (Krude et al. 2003; Smart and Low 2003).

Activation of α3β4 nAChRs on POMC neurons leads to neuronal activation and the release of melanocortin-4 agonists that activate MC4R in the PVN, inhibiting food intake, but such an effect is not observed in POMC KO mice (Mineur et al. 2011). Nicotine increases the firing rate of POMC neurons and decreases food intake in wild-type mice, but no significant difference was observed in food intake in response to nicotine or cytosine in POMC KO mice (Mineur et al. 2011). In addition, in mice, nicotine treatment increases the hypothalamic concentration of cocaine–amphetamine-regulated transcript- (CART-) and POMC-derived α-MSH and reduces NPY and AgRP availability (Chen et al. 2006; Marangon et al. 1998). Nicotine induces weight loss in both humans and rodents, although it increases the firing of both POMC- and NPY-containing neurons in the ARC (Huang et al. 2011).

Neurons expressing anorexigenic POMC and CART interact with orexigenic NPY- and AgRP-expressing neurons. Activation of POMC- and NPY-containing neurons, regarded as the two main neuronal populations in the hypothalamus, stimulates or inhibits neuronal feeding pathways, respectively (Picciotto and Mineur 2014). Hypothalamic POMC and AgRP mRNA are downregulated in the dietarily obese mouse, consistent with the anorexigenic effect of POMC and AgRP, but NPY mRNA expression also is reduced (Lin et al. 2000; Wang et al. 2002). Although diet can trigger the suppression of orexigenic mechanisms at the transcription level, the orexigenic effect also is initiated to sustain energy homeostasis.

3.3 MC4R

The melanocortins decrease food intake mainly through MC3R and MC4R. The melanocortin system is critical for the regulation of food intake and energy expenditure (Horvath et al. 2004). MC4R activation by melanocortins exerts effects on feeding behavior and energy homeostasis (Chen et al. 2012; Tao 2010). MC4Rs are widely distributed in the CNS, including some nuclei in the hypothalamus, such as the PVA, dorsomedial nucleus, and lateral hypothalamic area (LHA), contributing to the regulation of energy balance, sympathetic outflow, and glucose metabolism

(Kishi et al. 2003). In ARC, the MC system is a key relay for POMC- and AgRP-expressing neurons in regulating energy balance and body weight (Cone 2005; Schwartz et al. 2000). In humans and rodents, dysfunction of MC4R contributes to the development of obesity, insulin resistance, and diabetes (Huszar et al. 1997; Vaisse et al. 2000).

POMC neurons transmit the signal of nicotine activation to MC4Rs on secondary neurons to suppress appetite. Delivering shRNA by AAV to knock out expression of MC4R in the PVN significantly blocks nicotinic-induced hypophagia, confirming that both melanocortins and MC4R are critical to feeding regulation (Mineur et al. 2011). Moreover, there is an interaction between the opioid and melanocortin systems, corroborated by the observation that agonists for MC3R and MC4R blunt the orexigenic effect of β-endorphin (Grossman et al. 2003). Accordingly, the orexigenic effect of an MC3R/MC4R antagonist is suppressed by a selective MOR antagonist (Grossman et al. 2003).

3.4 AgRP

AgRP is another orexigenic molecule that co-localizes with NPY in the hypothalamus. Neuronal subpopulations, including AgRP-, NPY-, and POMC-containing neurons, make up the main central sensor of energy storage in the ARC, lying beside the ventral part of the third ventricle (Cone 2005). Of note, AgRP and α-MSH share targets, MC3R and MC4R, the two main receptors for melanocortin, to decrease food intake. Naloxone reduces AgRP-induced feeding stimulation (Hagan et al. 2001; Olszewski et al. 2001). On the other hand, AgRP and α-MSH are the antagonist and agonist for these receptors, respectively (Zoli and Picciotto 2012). AgRP/NPY neurons project onto POMC-expressing neurons and inhibit their activity by releasing NPY, AgRP, and GABA (Zoli and Picciotto 2012).

Orexigenic AgRP- and NPY-expressing neurons cooperate synchronously with anorexigenic POMC- and CART-expressing neurons to maintain the balance between orexigenic and anorexigenic neuropeptides. The blockade of both MOR and KOR suppresses the orexigenic action of AgRP, indicating that the opioid receptors are responsible for feeding behavior (Brugman et al. 2002). In addition, both AgRP and opioids show an effect, not only on food intake but also on food selection; and AgRP-induced feeding behavior depends on interaction with opioid receptors (Hagan et al. 2001).

Nicotine treatment suppresses AgRP in the mouse hypothalamus (Chen et al. 2006; Martinez de Morentin et al. 2012), while nicotine withdrawal is related to increased AgRP (Fornari et al. 2007), leading to a greater desire to eat. Both AgRP and NPY in the ARC not only induce food intake but also increase food reward (Fulton 2010), confirming that, apart from the effect of peripheral homeostatic signals on food reward circuits, the integration of neuronal homeostatic signals influences the reward circuits. Insulin receptor KO in mice on AgRP neurons reduces the ability of insulin to suppress the production of hepatic glucose (Shin

et al. 2017), suggesting that orexigenic AgRP is a key mechanism of insulin and energy homeostasis.

4 Peripheral Hormones

4.1 Ghrelin

Ghrelin, a 28-amino acid peptide synthesized and secreted in the stomach and hypothalamus, enhances appetite and induces deposition of fat in rodents. This hormone can increase appetite and food intake and exert an effect on body weight and fat mass deposition through a specific receptor (Wynne et al. 2005). Ghrelin is related to the hypothalamo-pituitary growth axis (Kojima et al. 1999), and many studies have revealed the physiological roles of circuiting ghrelin on appetite and food intake in rodents as well as in humans (Egecioglu et al. 2010; Wren et al. 2001). In addition, meal initiation can be regulated by ghrelin, and higher ghrelin concentrations are associated with greater hunger scores in healthy humans (Cummings et al. 2004).

Ghrelin receptors are possible pharmacological targets for reversing obesity (Engel and Jerlhag 2014), as the peptide increases adiposity by reducing fat expenditure, and blocking them may have the opposite effect (Tschop et al. 2000). Hypothalamic ghrelin receptors (GHS-R1A) contribute to ghrelin's effect on food intake, fat accumulation, and energy balance (Cowley et al. 2003). Interestingly, in rodents, ghrelin and its receptors have roles in several other physiological processes as well, such as regulating the serum glucose concentration, increasing prolactin secretion, inducing sleep, regulating the cardiovascular system (van der Lely et al. 2004), and stimulating gastric motility (Masuda et al. 2000). In addition, ghrelin correlates with memory formation via hippocampal GHS-R1A (Diano et al. 2006) and anxiety- and depressive-like behavior in rodents (Hansson et al. 2011).

18-Methoxycoronaridine (18-MC), a selective antagonist of α3β4 nicotinic receptors, prevents drug abuse (Pace et al. 2004) and attenuates sucrose intake (Taraschenko et al. 2008). The findings that treatment with 18-MC (20 mg/kg intraperitoneally) blocked ghrelin-induced increases in 5% sucrose intake and that 18-MC attenuates ghrelin-induced increases in extracellular dopamine within the ARC suggest that 18-MC or α3β4 nAChRs exerts its effects on feeding and food reward via modulation of ghrelin (McCallum et al. 2011). This appetite-stimulating hormone is not only highly expressed in the hypothalamus but is distributed widely in the mesolimbic dopamine system (Engel and Jerlhag 2014). In addition to being key in regulating food intake, hypothalamic ghrelin receptors (GHS-R1A) function in reward circuits (Engel and Jerlhag 2014). Ghrelin induces feeding behavior in the mesolimbic reward pathway between the ventral tegmental area (VTA) and the ARC (Abizaid et al. 2006; Naleid et al. 2005). The cholinergic–dopaminergic reward link is an important part of reward systems and assists in the reinforcing properties of natural rewards and addictive drugs (Larsson and Engel 2004;

Soderpalm and Ericson 2013). Ghrelin administration directly into the VTA or lateral dorsal tegmental nucleus (LDTg) increases accumbal dopamine release and locomotor stimulation. Furthermore, peripheral or intra-LDTg ghrelin administration increases VTA acetylcholine and accumbal dopamine release, and the effect of peripheral ghrelin can be blocked by a GHS-R1A antagonist (Jerlhag et al. 2012). GHS-R1As are expressed on both dopaminergic neurons in the VTA (Abizaid et al. 2006) and cholinergic cells in the LDTg (Dickson et al. 2010). Circulating ghrelin can pass the blood–brain barrier (Banks et al. 2002), and peripheral ghrelin administration increases accumbal dopamine release (Banks et al. 2002). Ghrelin enhances food rewards by interacting with NPY Y1 and opioid receptors in mice (Skibicka et al. 2012).

As noted, ghrelin, an endocrine signal that modulates the mesocorticolimbic dopaminergic system, is associated with the rewarding effects of food and drugs of abuse. With 26 healthy normal-weight never smokers, nicotine administration decreased correlations with ghrelin concentrations in the mesocorticolimbic system during fasting, whereas it increased the modulatory effects on food-cue reactivity after an oral glucose test (Kroemer et al. 2015). The findings that mecamylamine suppresses food intake induced by ghrelin and attenuates the ability of palatable food to condition a place preference suggest a modulatory effect of nAChRs on ghrelin-induced food intake and the rewarding properties of food (Dickson et al. 2010). However, although a sharply decreased body weight was observed during four weeks of cigarette smoke exposure, no significant difference was seen in the serum ghrelin concentration between the two groups (Ypsilantis et al. 2013).

α3β2, β3*, and α6* nAChRs expressed in the VTA have critical roles in ghrelin-induced reward (Jerlhag et al. 2008). The finding that the unselective nAChRs antagonist mecamylamine blocks the ability of ghrelin injected into the LDTg to increase dopamine in the ARC, but not acetylcholine in the VTA, shows that ghrelin stimulates the cholinergic–dopaminergic reward link (Jerlhag et al. 2012). One study investigated the ability of ghrelin to produce smoking cessation, which showed that a higher ghrelin concentration may increase the risk of smoking relapse (al'Absi et al. 2014). This finding provides confirmation of the effect of ghrelin on drug-induced reinforcement. Another study found that early tobacco withdrawal is associated with a significantly higher plasma concentration of the orexigenic peptide acetylated ghrelin, and there was a significant negative correlation between acetylated ghrelin and the Fagerström Test for Nicotine Dependence (FTND) or cigarettes per day (CPD) scores (Koopmann et al. 2015).

4.2 Leptin

Leptin, a 16-kDa non-glycosylated protein, was discovered in WAT (Zhang et al. 1994). This periphery-derived circuiting hormone is an important energy homeostasis-regulating factor that triggers reduction in food intake and increases energy consumption by regulating anorexigenic and orexigenic factors (Rosenbaum

and Leibel 2014). Exogenous administration of leptin suppresses expression of NPY in leptin-deficient obese (ob/ob) mice (Schwartz et al. 1996). In addition, leptin inhibits AGRP release and increases the firing rate of POMC neurons in the ARC (Breen et al. 2005). In contrast, CART, CCK, MC4-R, and CRH (inhibitors of food intake) are upregulated by leptin (Elmquist et al. 1998). On the other hand, the synthesis of leptin is affected by food intake, other feeding-related hormones, energy status, sex hormones, and inflammatory mediators (Gualillo et al. 2000).

Leptin exerts its biological functions by binding to a specific receptor, LEPR, which belongs to the class I cytokine-receptor superfamily and is present in both soluble and long isoforms (LepRB). The long isoform is the main leptin receptor for regulating food intake and is highly expressed in the mediobasal hypothalamic "satiety" (ARC) and "feeding" (LHA) centers (Leinninger et al. 2009). This receptor probably transduces extracellular leptin signals through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways (Fruhbeck 2006). Beyond the effect of leptin on feeding, Pelleymounter et al. (1995) provided direct evidence that leptin enhances oxygen consumption in ob/ob mice. Furthermore, Collines et al. (1996) observed that leptin administration increases norepinephrine in the brown adipose tissue (BAT) in ob/ob mice, indicating that the action of leptin on energy expenditure is associated with higher thermogenesis in BAT.

The results from human studies of nicotine effects on plasma leptin concentrations are inconsistent. Epidemiological studies showed significantly lower concentrations in smokers, even after adjusting for BMI (Hodge et al. 1997; Wei et al. 1997). However, in another study, plasma leptin was higher in smokers (Eliasson and Smith 1999). In addition, no change was observed in the concentration after a week's nicotine abstinence under controlled dietary conditions (Oeser et al. 1999). Consistent with the reduced leptin in smokers, Li and Kane (2003) observed significantly lower leptin RNA in fat tissue and plasma leptin in nicotine-treated rats compared with saline-treated controls. They also detected a dose-dependent correlation between plasma leptin and nicotine concentration (Li and Kane 2003). In another study, although smokers had lower leptin concentrations, the correlation of smoking with leptin became nonsignificant after adjusting for anthropometric parameters (Hussain et al. 2012). Those investigators also observed that leptin correlates significantly with BMI and waist circumference (Hussain et al. 2012).

Serum leptin is decreased in rats after both cigarette smoke exposure and cessation in plexiglass chambers (Ypsilantis et al. 2013). The plasma leptin concentration is decreased by 34%, in line with the decreased fat mass in balb/c mice exposed to cigarette smoke (Chen et al. 2005). Nicotine also reduces the leptin concentration in normal-fat-fed C57BL/6 mice, whereas high-fat-diet-induced obese mice show a blunted leptin response to nicotine (Hur et al. 2010). In addition, during fasting, nicotine administration by gum to healthy normal-weight never smokers increases the correlation with leptin concentration in the mesocorticolimbic system compared with ghrelin (Kroemer et al. 2015).

4.3 PYY

PYY, a 36-amino acid anorexic hormone, is secreted primarily by L cells in the gastrointestinal tract in response to eating and suppresses food intake (Valassi et al. 2008). PYY KO mice develop hyperphagia, and acute administration of PYY ameliorates this abnormality (Batterham et al. 2006). Also, the endogenous PYY concentration is lower in obese animals and humans (Batterham and Bloom 2003), and PYY administration reduces food intake and body weight independent of obesity status (Batterham and Bloom 2003; Batterham et al. 2003, 2002). Circulating PYY increases satiety and thus reduces food intake via gut–brain communication (Batterham et al. 2002; Karra and Batterham 2010). The hormone also inhibits pancreatic hormone secretion (Batterham et al. 2002) and gastrointestinal motility (Imamura 2002). However, the relation between circulating PYY and adiposity is controversial (Boggiano et al. 2005).

PYY also may be involved in regulating the rewarding effects of drug use and abuse (Schloegl et al. 2011). Similar to the acute effects of many drugs of abuse, PYY stimulates neurons in the mesoaccumbens dopaminergic pathways, either directly or indirectly (Al'Absi et al. 2014), and interacts with other neuropeptides linked to the rewarding effects of both food and abused drugs in multiple brain regions (Volkow et al. 2012). In the hypothalamic ARC, PYY inhibits NPY neurons and activates POMC neurons and may therefore impact affective and reward-related processes (Batterham et al. 2002; Challis et al. 2003). This was demonstrated in a study in which KO mice lacking PYY exhibited enhanced anxiety and depressive-like behaviors (Painsipp et al. 2010).

Guillermo et al. (1996) reported that nicotine treatment resulted in an elevation of PYY peptide concentrations in the ileum but a decrease in the colon. However, two recent studies did not observe a significant change in PYY in 22 nonobese adult male smokers (Pankova et al. 2016) and 27 long-term smokers after 3 months of smoking abstinence (Stadler et al. 2014), although in both, body weight showed a significant increase. It is possible that the PYY concentration is not regulated entirely by identical mechanisms in different body parts. Elevated PYY is associated with a lower desire to avoid withdrawal symptoms but not with relapse when the ability of PYY to predict smoking relapse was investigated during the initial 24–48 h of smoking cessation (Al'Absi et al. 2014). However, in another study, the same investigators found that PYY increases significantly in persons who relapse during the initial 48 h of a smoking cessation attempt (Lemieux and Al'Absi 2017).

5 Concluding Remarks

Epidemiology data confirm the inverse association between cigarette smoking and body weight. In spite of the widespread knowledge of the serious consequences of cigarette smoking and public efforts to curtail smoking onset and promote

cessation, a large number of persons continue to smoke, partly because they view their habit as a method of weight control. Beyond the addictive properties of nicotine, weight gain after smoking cessation is a contributor to the failure of smoking cessation efforts. Over the last decades, many studies have aimed to reveal the molecular and cellular mechanisms underlying nicotine's ability to decrease food intake. This chapter summarizes the best-documented central and peripheral transmitters and hormones involved in this effect.

We focused primarily on NPY, POMC, MC4R, and AgRP of the nervous system and leptin, ghrelin, and PYY of the periphery. Among these peptides, NPY, AgRP, and ghrelin stimulate food intake, whereas the others are anorexic hormones that inhibit appetite and food consumption. Although chronic administration of nicotine could result in upregulation of anorexigenic hormones and downregulation of orexigenic hormones, inverse results have also been observed. The complex interplay between neurons and appetite-regulating peptides has led to these inconsistent results. Moreover, appetite-regulated peptides such as other nAChRs, orexin, CCK, GLP-1, adiponectin, interleukin-6, UCP, and small-molecule neurotransmitters may participate in the interaction network of appetite regulation. In fact, the effects of nicotine on body weight cannot be explained simply by its influence on food intake and energy expenditure; social environmental factors, psychological factors, and genetics probably also play roles.

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Chapter 16
Nicotine Modulates Innate Immune Pathways via α7 Nicotinic Acetylcholine Receptor

Abstract Nicotine exerts its anti-inflammatory effects on multiple cell types and may benefit neurons in various degenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), in which an inflammation-related mechanism is implicated. Among the various nAChRs, α7, which has been identified in both neurons and immune cells and has high permeability to calcium, is believed to contribute significantly to nicotine's anti-inflammatory and neuroprotective effects. Although nicotine has been explored clinically for the treatment of some inflammatory diseases such as ulcerative colitis, the molecular mechanisms of its actions are largely unknown. In this chapter, we provide current evidence for nicotine's modulation of multiple immune pathways via α7 nAChRs in both neurons and immune cells. Understanding the mechanism of the nicotinic anti-inflammatory effect and neuroprotective function may guide development of novel medicines to treat infectious and neurodegenerative diseases.

Keywords Nicotinic acetylcholine receptor · α7 subunit · Innate immune pathways · Inflammation · Lipopolysaccharide · Jak/STAT pathway · Toll-like receptors (TLRs) · α-bungarotoxin · Poly(I:C)

1 Introduction

Although tobacco addiction has been a focus of research for years, the relation between smoking and inflammation-related diseases has not received much attention until recently. As an important component of tobacco smoke, nicotine is responsible for addiction and its related diseases. On the other hand, nicotine shows certain beneficial effects on mammalian cognition. For example, nicotine improves the cognitive performance, especially the attentional performance, of healthy nonsmokers, AD patients, and schizophrenics (Barr et al. 2008; Dalack et al. 1998; Glassman 1993; Kumari et al. 2003; Sahakian et al. 1989; Sahakian and Coull 1994). Both acute and chronic nicotine treatment improve working memory in animal models (Rezvani and Levin 2001). The cognitive defect in AD is related to inflammation in the brain, and nicotine exposure dampens the inflammatory

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responses to myelin antigens in a mouse model, suggesting a therapeutic effect (Shi et al. 2009).

Similarly, in the peripheral nervous system (PNS), the therapeutic anti-inflammatory potential of nicotine has been documented in epidemiological studies. One of the most compelling pieces of evidence is that about 90% of patients suffering from ulcerative colitis, a characteristic inflammatory bowel disorder, are non-smokers; and patients who have a history of smoking may get the disease after smoking cessation (Pullan et al. 1994; Rubin and Hanauer 2000; Thomas et al. 2005). Chronic obstructive pulmonary disease (COPD), which is caused by lung infections and favored by nicotine's sustained anti-inflammatory potential, is more common among smokers (Nuorti et al. 2000); and smoking cessation is the only strategy that reduces the resulting decline in lung function (Barnes 2003). Together, these clinical findings suggest that nicotine can exert pharmacological anti-inflammatory actions in both the central nervous system (CNS) and the PNS. This chapter provides an updated view of the current understanding of the mechanism of nicotine's regulatory effect on the innate immune responses at the level of molecular pathways.

2 Nicotinic Receptors and Their Involvement in the Immune System

Nicotine has various biochemical and pharmacological effects, ranging from cell adhesion to reduction of inflammation, which it exerts by interacting directly with nAChRs on cell membranes. The nAChRs are ion channels composed of five identical or homologous subunits, in which the ligand-binding sites are buried (Fig. 16.1a). Each subunit is constructed of four hydrophobic transmembrane segments (M1–M4) and two hydrophilic loops, one connecting M1–M2 and the other connecting M2–M3 (Fig. 16.1b), the whole having a molecular mass of about 290 kDa (Kalamida et al. 2007). These proteins are found on both peripheral and central neurons in the immune system and on other non-neuron cells, such as those of the skin (Albuquerque et al. 2009). Since the existence of the receptive substance of nicotine was reported in 1905 (Langley 1905), 17 types of subunits of nAChRs have been identified in vertebrates and designated α1–α10, β1–β4, γ, δ, and ε. For a detailed description of the genes encoding those nAChR subunits and their evolutionary relationship, please see Chap. 18.

Different types of nAChRs have different affinities for nicotine. The high-affinity receptors usually contain α4 and β2 subunits (Flores et al. 1996; McCallum et al. 2006). In the CNS, there are two principal classes of nAChRs: one with low affinity for nicotine that is α-bungarotoxin (α-Bgtx) insensitive, the other with high affinity for nicotine that is α-Bgtx-sensitive (Gotti and Clementi 2004). The α-Bgtx-sensitive nAChRs are the α7, α8, α9, and α10 subunits and can be either homomeric or heteromeric, whereas the α-Bgtx-insensitive receptors are made up of α (α2–α6) and β (β2–β4) subunits and are always heteromeric.

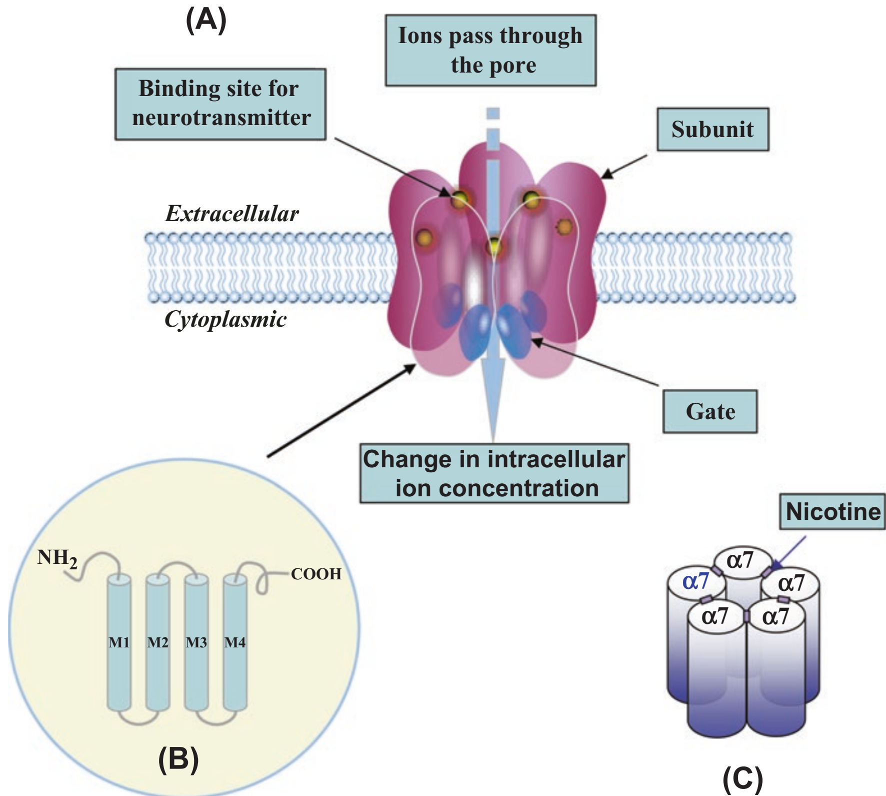

Fig. 16.1 Structure of nicotinic acetylcholine receptors (nAChRs). (**a**) nAChR is located on the cytoplasmic membrane and consists of five subunits, in which the ligand-binding site is buried. The number of binding sites differs for different receptor subunits. Neuronal nAChR is an ion channel permeable to cations such as Na^+ and Ca^{2+} in multiple cell types. (**b**) Each nAChR subunit is constructed of four hydrophobic transmembrane segments (M1–M4) and two hydrophilic loops connecting M1–M2 and M2–M3. (**c**) α7 nAChR is composed of five homomeric subunits

Different subunits usually are involved in different conditions. For example, α3 functions in palmoplantar pustulosis and autoimmune autonomic ganglionopathy; β2 is related to pain, heart disease, addiction, epilepsy, schizophrenia, PD, and AD; and α7 functions in the immune system (D'Hoedt and Bertrand 2009). Multiple subunits such as α7, β2, and β4 are found in different types of immune cells derived from the bone marrow, especially macrophages (Galvis et al. 2006; Wang et al. 2003), which are key to the immune response. Nicotine suppresses the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), in activated macrophages to a more significant extent than does the same dose of acetylcholine (Wang et al. 2003). By interaction with the α7 homopentamer nAChR (Drisdel and Green 2000; Rangwala et al. 1997), whose structure is illustrated in Fig. 16.1c, nicotine can regulate the production of pro-inflammatory cytokines, not only tumor

necrosis factor (TNF)-α but also interleukin (IL)-1 and IL-1β (Wang et al. 2003). Although other types of nAChRs, such as α5-containing nAChR (Orr-Urtreger et al. 2005), also are implicated in nicotine's anti-inflammatory effect, attention has been drawn to α7 nAChR because of its crucial role in regulating the immune responses of various cell types (Aicher et al. 2003; Guinet et al. 2004; Nouri-Shirazi and Guinet 2003; Skok et al. 2006; Suzuki et al. 2006). However, because limited information is available and the exact effect and mechanism are largely unknown, nAChRs other than α7 nAChR will not be discussed in this chapter.

3 Regulatory Effect of Nicotine on Innate Immune-Related Pathways

3.1 Inhibition by Nicotine of Inflammation in Activated Immune Cells

The immune system is highly developed to protect the host from invading pathogens, in part, by producing pro-inflammatory and anti-inflammatory cytokines. When this system is not properly regulated, immune disorders cause harm. These disorders can be categorized into two groups: autoimmune and inflammatory. Autoimmune diseases are caused by aberrant responses to the host's own proteins, leading to "self-attacks," whereas inflammatory diseases are caused by excessive immune responses. Properly regulating the immune response is therefore crucial for preventing and treating immune disorders. Recently, cholinergic pathways were found to exert anti-inflammatory effects in multiple cell types, contributing to immune control (Saeed et al. 2005; Sugano et al. 1998; Wang et al. 2004). These pathways react more rapidly and locally than the anti-inflammatory cytokines and pituitary–adrenal glucocorticoids previously used to tamp down immune reactions.

In the peripheral system, α7 is the essential subunit for acetylcholine inhibition of lipopolysaccharide (LPS)-induced release of pro-inflammatory cytokines, such as TNF in macrophages at the posttranscriptional stage, without affecting the expression of anti-inflammatory cytokines such as IL-10 (Wang et al. 2003). α7-deficient macrophages fail to respond to nicotine or acetylcholine under endotoxin stimulation (Borovikova et al. 2000; Wang et al. 2003). In agreement with this finding, LPS-induced release of high-mobility group box chromosomal protein 1 (HMGB1) and activation of nuclear factor (NF)-κB, a key mediator of inflammatory responses that induce transcription of pro-inflammatory cytokines in activated immune cells (Baeuerle and Henkel 1994; Li and Verma 2002) can be inhibited by nicotine via α7 nAChR, whose antagonist can neutralize nicotine's suppressive effect on NF-κB in macrophages in a dose-dependent manner both in vivo and *in vitro* (Wang et al. 2004). These conclusions are supported by the finding that GTS-21, a selective α7 nAChR agonist, improves survival in murine endotoxemia and severe sepsis (Pavlov et al. 2007). Nicotine also suppresses the expression of LPS-induced macrophage inflammatory protein (MIP)-1α and MIP-1β at the mRNA level in human monocytes (Yoshikawa et al. 2006).

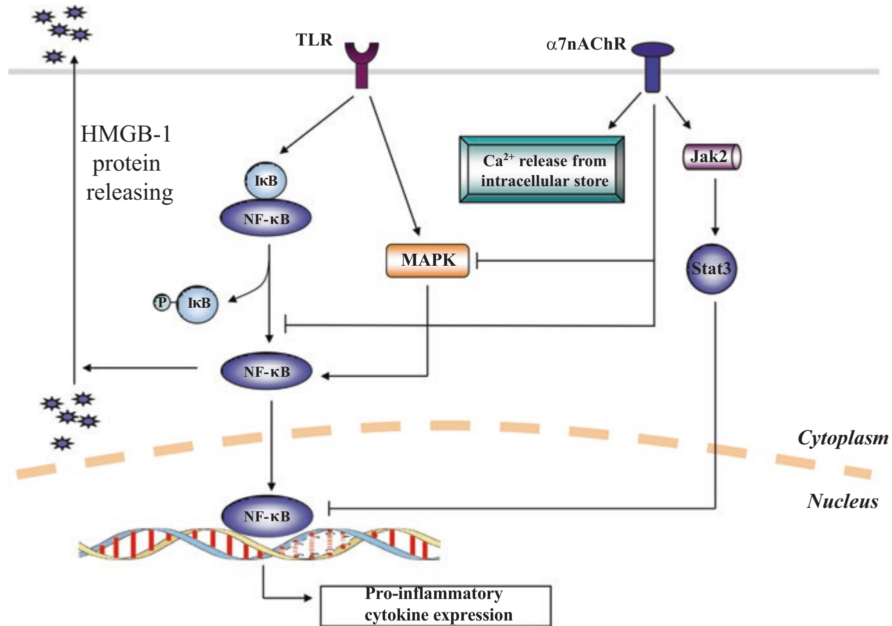

Fig. 16.2 Nicotine's regulatory effect on immune-related pathways. Nicotine exerts its anti-inflammatory effect in activated immune cells, such as macrophages and microglia, by interacting with α7 nAChR. Activated α7 nAChR binds directly to Jak2 and triggers the Jak2/STAT3 pathway to interfere with activation of TLR-induced NF-κB, which is responsible for pro-inflammatory cytokine transcription. Activation of α7 nAChR also can suppress IκB phosphorylation and lead to MAPK activation and release of HMGB1 from the cytoplasm, along with its anti-inflammatory effects

In the CNS, microglia are the primary cells involved in local innate immune responses when activated by acute or chronic insults (Benveniste 1997; Gehrmann et al. 1995; Kreutzberg 1995). They usually are resting in normal healthy brain but release cytokines and free radicals after activation (Kreutzberg 1996; Nimmerjahn et al. 2005; Streit 2002). The local inflammatory state sustained by microglia may be the underlying mechanism of some neurodegenerative diseases, such as AD and PD. Nicotine may have a protective action against those diseases (Streit 2002; Wang et al. 2000a, b). Nicotine also suppresses LPS-induced TNF release in murine-derived microglial cells via α7 nAChR, and this effect is neutralized by α-Bgtx, a selective α7 antagonist (Shytle et al. 2004).

The signaling pathways involved in nicotine's anti-inflammatory effects have been investigated extensively (Borovikova et al. 2000; de Jonge et al. 2005; Guarini et al. 2003; Saeed et al. 2005; Siegel et al. 2007; Sugano et al. 1998). As shown in Fig. 16.2, there are multiple innate pathways implicated in these anti-inflammatory actions. Both p44/42 and p38 mitogen-activated protein kinase (MAPK) are necessary to the effects in microglial cells (Shytle et al. 2004). In macrophages, tyrosine kinase Jak2 interacts directly with nicotine-activated α7 nAChR and then phosphorylates signal transducer and activator of transcription 3 (STAT3) (de Jonge

et al. 2005), a key component of the anti-apoptosis cascade that modulates the *trans*-active response mediating the anti-inflammatory process (Takeda et al. 1999; Welte et al. 2003). Nicotine's suppressive effect on pro-inflammatory cytokine expression is dependent on phosphorylated STAT3, which forms dimers and translocates into the cell nucleus for involvement in gene transcription. Although STAT3 is not related directly to pro-inflammatory cytokine production, the attenuated cytokine expression mediated by α7 nAChR may be caused by the collaboration of NF-κB and the Jak/STAT pathway (de Jonge and Ulloa 2007). This process can be blocked by the α7-selective antagonist α-Bgtx, as well as by methyllycaconitine (MLA) and AG490, a selective inhibitor of Jak2 phosphorylation. Vagus nerve stimulation does not reduce peritoneal cytokine concentrations or intestinal inflammation in STAT3-deficient macrophages, whereas it does in the wild type. Nicotine also can induce the expression of suppressor of cytokine signaling 3 (SOCS3), which negatively regulates cytokine signaling through the Jak/STAT pathway. However, nicotinic inhibition of macrophage activation is not dependent on the expression of this protein (de Jonge et al. 2005). The suppressive effect of nicotine on NF-κB is mediated by inhibition of NF-κB inhibitor (IκB) phosphorylation without affecting IκB expression, interfering with nuclear translocation of NF-κB via α7 nAChR; it can be neutralized by α-Bgtx (Yoshikawa et al. 2006). HMGB1 was first identified as a transcription and growth factor, but recently, it was found to act also as a pro-inflammatory cytokine, inducing expression of TNF, IL-1β, and IL-6 and mediating inflammation in various diseases, such as sepsis (Andersson et al. 2000; Bustin 2002; Li et al. 2003; Wang et al. 1999; Yang et al. 2004). Its sequence is highly conserved evolutionarily (Ferrari et al. 1994; Paonessa et al. 1987; Wen et al. 1989) and is expressed in almost all types of cells, although the extent of expression can be different. The release of HMGB1 is controlled by NF-κB, and nicotine suppresses HMGB1 secretion by reducing the activity of NF-κB. However, the total protein and cytoplasmic mRNA quantities of this cytokine are not affected (Wang et al. 2004), and nicotine does not induce degradation of extracellular HMGB1. This mechanism is strikingly different from that of nicotinic regulation of LPS-induced production of the pro-inflammatory cytokines TNF, IL-1β, and IL-6.

Toll-like receptors (TLRs) are one of the most important families in the innate immune system, mediating inflammatory processes induced by various pathogens (Beutler 2002, 2003a, b; Hoebe et al. 2003). There are two pathways downstream of TLR receptors that are dependent on different adaptors, namely, Toll-like receptor adaptor molecule 1 (TRIF) and myeloid differentiation primary response gene 88 (*MyD88*). Activation of the TLR pathway is dependent on intracellular calcium signaling (Liu et al. 2008). Triggering the MyD88- or TRIF-dependent pathway can induce the release of intracellular calcium stores and thus increase the concentration, which promotes phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII), resulting in the production of pro-inflammatory cytokines and type I interferon (IFN-1). Interestingly, activation of nAChRs suppresses LPS-induced inflammation in monocytes/macrophages in a calcium-dependent manner (Blanchet et al. 2006). Although neuronal nAChRs function predominantly as ion (mainly Ca^{2+}) channels (Albuquerque et al. 1995), in immune cells, they usually act through allosteric changes rather than as ligand-gated ion channels (Hecker et al.

2009; Razani-Boroujerdi et al. 2007). Activation of nAChRs in monocytes/macrophages can limit the LPS-induced calcium release dependent on PI3K and PLC and then suppress TNF production (Blanchet et al. 2006). In microglia, nicotine suppresses LPS-induced TNF in a PLC-dependent manner independent of extracellular Ca^{2+}, as no current was detected in LPS-stimulated microglial cells. Besides, JNK and p38 MAPK are suppressed by nicotine in the activated microglia, which are responsible for the posttranscriptional regulation of TNF (Suzuki et al. 2006).

Although great effort has been devoted to exploring the mechanism of nicotine's anti-inflammatory effect, and multiple pathways have been identified in the nicotinic anti-inflammation model, details of how nicotine regulates TLR pathways are still not clear. Nicotine can suppress the expression of CD14 and TLR4 in human blood monocytes either with or without stimulation by LPS (Hamano et al. 2006). To understand how nicotine regulates the TLR system, we used a real-time RT-PCR array containing a panel of 40 key genes from the pathway to identify the expression changes caused by nicotine at the RNA level. Given that TLR4 and TLR3 are the best-described receptors in the family, mediating responses to bacterial and viral infection, respectively, we used ligands to these two receptors to build an inflammation model.

In this study, RAW264.7 cells were stimulated with LPS 100 ng/ml or poly(I:C) 10 μg/ml with or without prior treatment with 5 μM nicotine. As shown in Fig. 16.3,

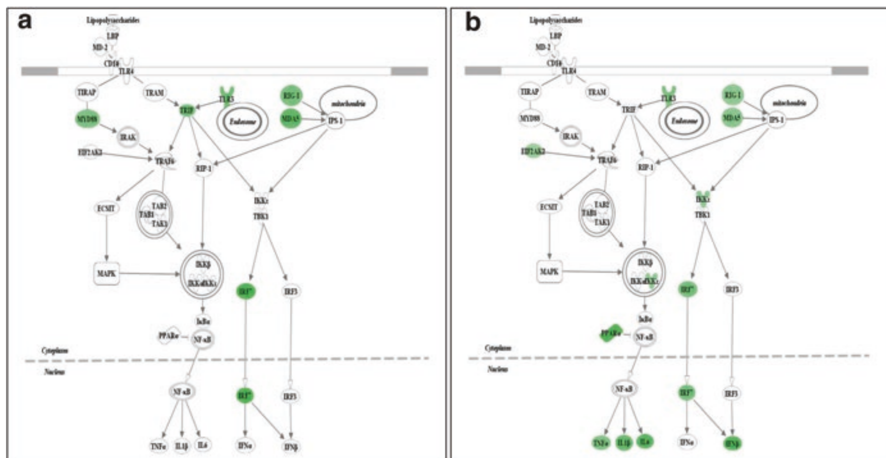

Fig. 16.3 Nicotine's effect on TLR pathways. (**a**) As a ligand for TLR4, LPS triggers both MyD88- and TRIF-dependent pathways, leading to activation of NF-κB, which is responsible for the transcription of various pro-inflammatory cytokines and type I interferons (i.e., IRF3). The MyD88-dependent pathway is activated early, whereas the TRIF-dependent pathway is activated in the late phase. The changes in RNA expression for more than 40 key representative genes in the TLR4 pathway in RAW264.7 cells were measured by real-time RT-PCR after the cells had been stimulated with LPS 100 ng/ml for 4 h with or without prior treatment with 5 μM nicotine for 30 min. (**b**) Poly(I:C) is a ligand for TLR3, triggering only the TRIF-dependent pathway. All results were obtained with RAW264.7 cells, which were stimulated with poly(I:C) 10 μg/ml for 16 h with or without prior treatment with 5 μM nicotine for 30 min. The genes significantly modulated by nicotine are indicated in green. Panels A and B were both constructed by Ingenuity Pathway Analysis software (www.ingenuity.com) with some modifications

expression of several genes is significantly suppressed by nicotine, indicating that both TLR pathways contribute to nicotine's anti-inflammatory effect, although it appears that nicotine modulates more genes in the TLR3 pathway. This pathway is TRIF dependent, whereas the TLR4 pathway depends on both TRIF and MyD88. Although it is likely that the TRIF-dependent pathway is more sensitive to nicotine, more experiments are needed to test this hypothesis.

3.2 Different Regulatory Effects of Nicotine on Resting Immune Cells

Long-term nicotine exposure can induce expression of TNF and inducible nitric oxide synthase (iNOS) in macrophage cell lines and contribute to an inflammatory state (Lau et al. 2006). In animal models, the quantity of serum inflammatory cytokines, including TNF, IL-1β, and keratinocyte-derived chemokine, is upregulated in response to nicotine (Lau et al. 2006). Another study showed that the production of TNF, interferon (IFN)-γ, and IL-6 is significantly decreased in spleen cells from α7-knockout (KO) mice (Fujii et al. 2007). Expression of TLR4 and CD14 is suppressed by nicotine in human blood monocytes (Hamano et al. 2006).

Because α7 nAChR is the major calcium channel, calcium signaling mediated by α7 nAChR has been studied, revealing that prolonged exposure to nicotine reduces expression of α7 nAChR and therefore suppresses the calcium signaling mediated by it. Accordingly, expression of α7 nAChR mRNA is significantly diminished in peripheral monocytes from smokers compared with non-smokers (Fujii et al. 2008).

3.3 Regulatory Effects of Nicotine on Neurons

In an inflammatory state, neuron injury usually is caused by the cytokines and chemokines released from nearby immune cells, such as microglia in the brain, or infiltrated into the CNS from the peripheral system. The immune-related function of nicotine is exhibited primarily as a suppressive effect on neuronal toxic component production by the immune cells in the CNS, as shown in Fig. 16.4. However, nicotine also can interact directly with neuronal cells to regulate some immune-related pathways, and its neuronal protective role has been confirmed by some independent experts (Kihara et al. 1997; Kincade et al. 2005). Nicotinic receptors are expressed throughout the nervous system and regulate expression of genes in different pathways (Dunckley and Lukas 2003, 2006; Gutala et al. 2006). Although the neuron has limited ability to release cytokines and chemokines to evoke the inflammation cascade, nicotine can trigger some immune-related downstream signaling pathways, especially the Ca^{2+}-dependent pathways such as phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II

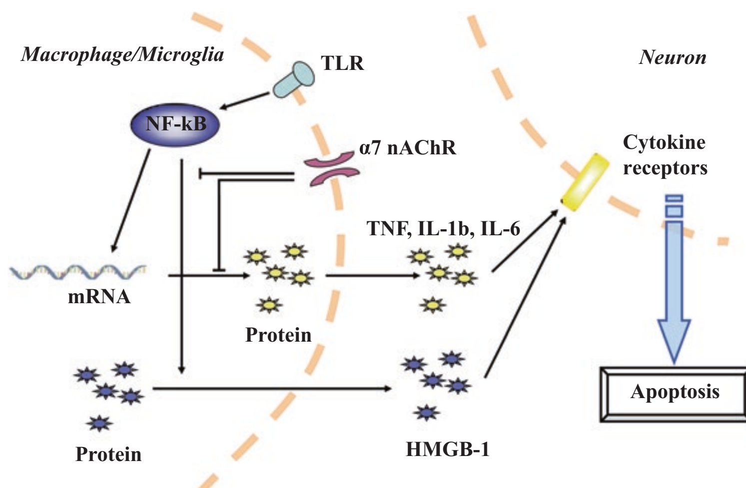

Fig. 16.4 Proposed major protection strategies for neurons by nicotine. Infiltrating macrophages and microglia in the brain can release many potentially neurotoxic components, such as proinflammatory cytokines and chemokines, as shown in this figure, resulting in neuron injury. The nicotinic anti-inflammatory pathway reduces activation of NF-κB to suppress the production of those compounds and protect neurons

(CaMKII), becoming involved in regulation of transmitter release, synaptic plasticity, and cell survival and providing benefit in some neurodegenerative diseases such as AD and PD (Damaj 2000; Hejmadi et al. 2003; Kihara et al. 2001; Liu et al. 2001; Messing et al. 1989; Picciotto and Zoli 2002; Shaw et al. 2002). Unfortunately, the indications and the precise dose that will produce desirable effects are not yet known.

Mammal neuronal nAChRs have long been known to mediate Na^+ influx, induce membrane depolarization, and activate voltage-gated Ca^{2+} channels (Colquhoun 1987; Derkach et al. 1983). The α7 nAChR, one of the most abundant nicotinic receptors in neurons, has well-known high calcium permeability (Albuquerque et al. 1995). In the early stage of α7 nAChR activation, phosphorylation occurs in many types of neurons. This event is triggered by Src family kinases (SFKs), which interact directly with the cytoplasmic loop of α7 nAChR and process the phosphorylation, reducing the activity of α7 nAChR, which can be dephosphorylated by tyrosine phosphatases. The activation of α7 nAChR is upregulated when the tyrosine kinase is inhibited, whereas inhibition of tyrosine phosphatases or mutation of the cytoplasmic tyrosine residue reduces the activity of α7 nAChR. The functioning of α7 nAChR is decided by the balance of those two kinds of enzymes (Charpantier et al. 2005). In SH-SY5Y cells, nicotine triggers the activation of ERK 1/2 via α7 nAChR. This process is dependent on calcium signaling and cAMP-dependent protein kinase (PKA) and can be blocked by α-Bgtx and PD98059, an inhibitor of MAP kinase–ERK kinase (MEK), but not by inhibitors of PKC, CaMKII, or PI3K (Dajas-Bailador et al. 2002). Nicotine also can trigger the activation of cAMP-responsive element-binding protein (CREB) (Berg and Conroy 2002), a transcription factor

controlling the expression of numerous genes involved in learning and memory, which is dependent on calcium influx and MAPK activity.

Amyloid beta (Aβ) peptide accumulation and local inflammation are two components of the histopathologic changes attributable to AD. In studies of AD, nicotine activates α7 nAChR and then transfers signals to PI3K and AKT via Jak2 in PC12 cells to exert a neuroprotective effect against Aβ-(1-42) amyloid (Shaw et al. 2002). In Aβ precursor protein (APP)-transgenic mice, nicotine decreases the accumulation of Aβ in the hippocampus and cortex (Hellstrom-Lindahl et al. 2004; Nordberg et al. 2002). This event also is mediated by α7 nAChR, which leads to reduced activity of MAPK, resulting in suppressed activation of NF-κB and c-Myc. The production of NO therefore is reduced (Liu et al. 2007).

Nicotine also modulates the expression of numerous genes in neurons (Dunckley and Lukas 2003, 2006; Gutala et al. 2006). To determine how nicotine regulates multiple immune-related pathways, we treated SH-SY5Y cells, which respond to TLR3 ligand and trigger production of IFN-γ (Zhou et al. 2009), with acute nicotine administration and measured the expression of more than 3000 genes using microarray technology. Six immune-related pathways were significantly modulated by nicotine, namely, TLR, ERK, p38, death receptor, PI3K/AKT, and IL-6 (Cui et al. 2012, 2013). This result confirmed that nicotinic strongly affects the immune response.

4 Concluding Remarks

Nicotine controls the expression of many cytokines through anti-inflammatory pathways and plays a protective role in neurons via α7 nAChR. Therefore, it could be a therapeutic target in a range of disorders. Nicotine decreases the production of pro-inflammatory cytokines such as TNF without affecting the expression of anti-inflammatory cytokines in exotoxin-activated immune cells, both *in vivo* and *in vitro*, and improves survival in animal sepsis models. Activated α7 nAChR interacts directly with Jak2, which triggers the phosphorylation of transcription factor STAT3. This Jak/STAT pathway may disturb the activity of NF-κB, which is responsible for the transcription of numerous cytokines. The phosphorylation of IκB also is interrupted by nicotine in the inflammation model, resulting in reduced NF-κB activity. Whether these anti-inflammatory pathways mediated by α7 nAChR are dependent on calcium influx is still not clear.

The expression of several genes in the TLR pathways is regulated by nicotine. Interestingly, nicotine's effect on immune-related pathways seems to be quite different in resting cells. Its function differs among cell types and by dose. In neurons, nicotine can trigger the activation of ERK 1/2, which mediates cell survival. This process is dependent on calcium signaling and PKA. Also, in animal models of AD, nicotine protects neurons against Aβ accumulation through the Jak/STAT and PI3K/AKT pathways via α7 nAChR and has a suppressive effect on the activity of NF-κB and the c-Myc oncogene and on the production of NO.

The US Food and Drug Administration has approved electrical stimulation of the vagus nerve, which triggers the cholinergic anti-inflammatory pathway, to treat refractory epilepsy and recurrent depression. The therapeutic potential of nicotine is still limited by its nonspecific effects. Although the nicotinic anti-inflammatory pathway promises a mechanism to control the release of pro-inflammatory cytokines and might therefore be a therapeutic approach to infectious disorders, unlike the vagus nerve, nicotine may target other cell types and organs and thereby be toxic. For example, nicotine can suppress the activation of NF-κB in macrophages, reducing the release of pro-inflammatory cytokines. However, when nicotine interacts with parenchymal cells, in which NF-κB protects against cytotoxic reagents, it results in cell death. Nicotine also interacts with other non-α7 nAChRs and can cause serious adverse effects. This double-sided sword of nicotine makes it difficult to predict its clinical value. Greater understanding of the pathways modulated by nicotine during its anti-inflammatory actions is important to develop its therapeutic potential or design a novel specific agonist to α7 nAChR to avoid collateral effects on other types of cells or on receptors one does not desire to stimulate.

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Chapter 17
DNA Methylation Analysis Reveals a Strong Connection Between Tobacco Smoking and Cancer Pathogenesis

Abstract Tobacco smoking is a well-documented risk factor for various cancers, especially those of the lung. To test our hypothesis that abnormal DNA methylation loci associated with smoking are enriched in genes and pathways implicated in the pathogenesis of cancer, we analyzed two sets of smoking-related methylated genes, in blood and buccal samples, as reported in 28 studies. By analyzing 320 methylated genes from 26 studies of blood samples, we found 57 enriched pathways associated with different types of cancer (false discovery rate [FDR] <0.05). Of these, 11 were also significantly overrepresented in the 661 methylated genes found in the 2 studies of buccal samples. We further found that the aryl hydrocarbon receptor signaling pathway plays an important role in the initiation of smoking-attributable cancer. Finally, we constructed a subnetwork of genes important for smoking-attributable cancer from the 48 nonredundant genes in the 11 oncogenic pathways. Of these, genes such as *DUSP4* and *AKT3* are well documented as being involved in smoking-related lung cancer. Taken together, these findings provide robust and systematic evidence in support of smoking's impact on the epigenome, which may be an important contributor to cancer.

Keywords DNA methylation · Cancer · Epigenetics · *DUSP4* · *AKT3* · Pathways · Epigenome-wide association study · EWAS · Blood · Buccal · Lung cancer · RAR activation · Actin cytoskeleton signaling · Aryl hydrocarbon receptor signaling · Hub genes · CpG islands · Risk

1 Introduction

Cigarette smoking is a common adverse behavior resulting in cancers (Vineis et al. 2004). Notably, there is a much higher risk of lung cancer among smokers, between five- and tenfold higher than that in the non-smoking population. In developed countries, smoking causes more than four of five cases of lung cancer (CDC 2010). A World Health Organization report states that there are approximately six million smoking-related deaths worldwide every year, with most of them being caused by cancer (WHO 2013).

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More than 60 carcinogens are present in cigarette smoke, including polycyclic aromatic hydrocarbons (PAHs), nitrosamines, and aromatic amines, all of which play crucial roles in tumorigenesis (Pfeifer et al. 2002). Nicotine is not only the chief addictive compound that causes smokers to continue their habit but also is genotoxic, contributing to the pathogenesis of cancer (Grando 2014). Most of these carcinogens require metabolic activation via DNA adducts that evoke mutations and epigenetic reprogramming, creating genomic instability and other adverse genetic alterations (Hecht 2003).

Genetic association studies revealed many variants that underlie smoking-attributable cancers (Amos et al. 2008; Hung et al. 2008; Thorgeirsson et al. 2008). One of the most robust findings is that variants in the *CHRNA5/A3/B4* cluster on chromosome 15q24–25.1 are significantly associated with both ND and lung cancer (see Chap. 5). However, current genetics-based evidence has not elucidated the mechanisms of smoking-associated cancers, so many researchers are focused on the role of smoking-associated DNA methylation.

Methylation, a reversible and heritable alteration in DNA reflecting methyl group attachment to nucleotides, affects a disease by altering either the transcriptional regulation of genes (Bell et al. 2011), alternative splicing (Laurent et al. 2010), or the integrity of the genome (Law and Jacobsen 2010). Changes in DNA methylation have an important role in the early stages of carcinogenesis (Jones et al. 2013; Teschendorff et al. 2012). Furthermore, multiple candidate gene-specific methylation (GSM) studies (Sundar et al. 2011) showed that aberrant DNA methylation in the promoter region of genes conveying susceptibility to cigarette smoking and the risk of cancer.

As high-throughput next-generational sequencing and array platforms emerge, our research approach and concept have changed from hypothesis-driven exploration to data-driven hypothesis generation (Pastrello et al. 2014). Many epigenome-wide association studies (EWASs) have revealed a large number of methylated loci that are associated with in utero consequences of either maternal smoking (Maccani and Maccani 2015) or smoking in adulthood (Gao et al. 2015). Sustained exposure to cigarette smoke is a cause of epigenetic reprogramming at a global level, as demonstrated by measuring the methylation of repetitive elements, such as those of Sat2 (Flom et al. 2011) and LINE-1 (Furniss et al. 2008).

2 Genes Enriched by Smoking-Associated DNA Methylation in Blood

To identify all reports on the association of cigarette smoking with alterations in DNA methylation, 1447 studies published prior to June 13, 2015, were retrieved from the PUBMED database. The keywords used were "smoking," "smoke," "tobacco," "nicotine," and "cigarette" and "methylation." All abstracts of these reports were reviewed to identify potentially eligible papers. We also manually checked the references in each paper for additional studies not indexed by PUBMED. To eliminate or minimize false-positive findings, we narrowed our selection to papers on genes having significant reported associations with smoking. Once

a paper met the inclusion criteria, the full text was reviewed to ensure the conclusion was in accordance with the results.

After rigorous and systematic screening, 28 epigenetic association studies consisting of 9 candidate GSM studies and 19 EWASs were selected, among which 26 studies were conducted on DNA extracted from whole blood and 2 on DNA from buccal cells. Of them, the 26 blood studies encompassed 17,675 samples. We found 320 smoking-associated DNA methylation-enriched genes with at least two independent pieces of evidence, which were included in the pathway-based analysis in the discovery stage of this project.

Among the genes from the blood, many had strong association signals with smoking in multiple replications, such as *AHRR*, *F2RL3*, *AKT3*, and *GFI1*. For example, *AHRR*, a tumor suppressor gene on chromosome 5p15.33, encodes a class E basic helix–loop–helix protein that dampens the translocation of AHR–ligand complex to the nucleus. Knockout of *AHRR* correlates with greater tumor cell invasiveness in many tissues, including those of the lung, colon, ovary, and breast (Zudaire et al. 2008). The *F2RL3* protein is related to platelet activation and coagulation, as well as to cell signaling. Epigenetic association studies (Shenker et al. 2013; Zhang et al. 2014) provide consistent evidence that *F2RL3* methylation predisposes to involvement in lung or colon cancer. By performing a genome-wide methylation analysis, Fasanelli et al. (2015) demonstrated that smoking-induced hypomethylation in *AHRR* and *F2RL3* contributes to the risk of lung cancer, evidence that specific altered methylation that can influence the link of smoking to cancer pathogenesis.

3 Overrepresented Pathways of Methylated Genes in Blood

To understand the influence of smoking on cancer from an epigenetic perspective, we conducted pathway-based analyses for 320 methylated genes identified using the bioinformatics tools of Ingenuity Pathway Analysis, which revealed 90 overrepresented biological pathways having an FDR Q value of <0.05. Of these, 57 pathways are associated with cancer. For example, the most significant pathway, "MSP-RON signaling" (FDR Q value 2.2×10^{-4}; see Table 17.1), regulates the activity of macrophages in response to inflammatory stimuli, which is related to epithelial and leukemic carcinogenesis (Yao et al. 2013). The second significant pathway, "RAR activation," is overrepresented by 12 genes (FDR Q value 3.7×10^{-4}) and is prominently associated with the genesis of cancer (Altucci et al. 2007).

As shown in Table 17.1, we further found that some of these overrepresented pathways cause vulnerability to a specific type of cancer, such as signaling in "non-small cell lung cancer," "small-cell lung cancer," "pancreatic adenocarcinoma," "renal cell carcinoma," "ovarian cancer," and "prostate cancer." In addition, many other overrepresented pathways are involved in the oncogenic process of various cancers, which include "signaling by rho family GTPases" and signaling in "actin cytoskeleton," "AMPK," and "ERK/MAPK."

Table 17.1 Overrepresented pathways underlying smoking-attributable cancer from blood samples (FDR <0.01)

Canonical pathway	No. of genes	P value	FDR
MSP-RON signaling pathway	8	6.17×10^{-07}	0.00022
RAR activation	14	2.04×10^{-06}	0.00037
Rac signaling	10	6.17×10^{-06}	0.00071
Actin cytoskeleton signaling	14	7.94×10^{-06}	0.00071
Aryl hydrocarbon receptor signaling	11	1.15×10^{-05}	0.00083
Signaling by Rho family GTPases	14	2.51×10^{-05}	0.0015
AMPK signaling	12	2.951×10^{-05}	0.0016
Renin-angiotensin signaling	9	6.03×10^{-05}	0.0028
Molecular mechanisms of cancer	17	7.41×10^{-05}	0.0030
CXCR4 signaling	10	0.00017	0.0058
ERK/MAPK signaling	11	0.00021	0.0058
HER-2 signaling in breast cancer	7	0.00021	0.0058
Thrombin signaling	11	0.00022	0.0058
HGF signaling	8	0.00027	0.0060
Relaxin signaling	9	0.00028	0.0060
Role of tissue factor in cancer	8	0.00033	0.0063
Non-small cell lung cancer signaling	6	0.00060	0.0096

4 Common Molecular Pathways in Blood and Buccal Samples

To validate the findings from blood samples, we conducted a similar pathway-based analysis for significantly methylated genes in the buccal samples, which revealed 32 common pathways in both blood and buccal tissue. Among them, 11 were associated with cancer (Table 17.2), including “RAR activation,” “actin cytoskeleton signaling,” “aryl hydrocarbon receptor signaling,” “signaling by rho family GTPases,” and “molecular mechanisms of cancer.”

This confirmation in two types of specimens shows that these common oncogenic pathways play important roles in the pathology of smoking-attributable cancer. Particularly, aryl hydrocarbon receptor signaling is crucial to the detoxification of the damaging components of cigarette smoke, including PAHs, nitrosamines, and aromatic amines (Novakovic et al. 2014). If there were aberrant modifications in this biological regulation, these toxic substances could directly influence the epigenetic profile of circulating blood cells or other tissues. Using mice lacking the aryl hydrocarbon receptor (AhR), several studies have shown that AhR regulates angiogenesis by activating vascular endothelial growth factor in the endothelium and inactivating tumor growth factor-β in the stroma (Tsay et al. 2013); both reactions encourage the proliferation of tumor cells by supplying both nutrients and oxygen. Abnormal smoking-related DNA methylation in the aryl hydrocarbon receptor signaling pathway probably induces DNA adduct formation, causing miscoding of the sequence of DNA (Fig. 17.1). With long-term smoking exposure, the

Table 17.2 Eleven overrepresented cancer-related pathways in both blood and buccal samples

Canonical pathway	Discovery sample (blood)			Validation sample (buccal)	
	No. of genes	P value	FDR	No. of genes	P value
RAR activation	14	2.04×10^{-06}	0.00037	13	0.008
Actin cytoskeleton signaling	14	7.94×10^{-06}	0.0007	13	0.019
Aryl hydrocarbon receptor signaling	11	1.15×10^{-05}	0.0008	11	0.004
Signaling by Rho family GTPases	14	2.51×10^{-05}	0.002	13	0.039
Molecular mechanisms of cancer	17	7.41×10^{-05}	0.003	28	1.55×10^{-05}
G-protein-coupled receptor signaling	12	8.51×10^{-04}	0.012	17	0.004
PTEN signaling	7	0.003	0.021	9	0.014
Axonal guidance signaling	15	0.004	0.025	22	0.020
Colorectal cancer metastasis signaling	10	0.004	0.025	13	0.036
GNRH signaling	7	0.005	0.025	9	0.021
Breast cancer regulation by stathmin1	8	0.012	0.049	12	0.020

The cutoff threshold of discovery samples was FDR <0.05 and that of validation samples was $P < 0.05$

DNA suffers persistent miscoding that triggers epigenetic changes in various vital oncogenes, such as *NOTCH1*, *ATK3*, *DUSP4*, *SMAD6*, and *SMARCA4*, which are part of the most important enriched pathways (Fig. 17.2) leading to carcinogenesis. Thus, the aryl hydrocarbon receptor signaling pathway probably is needed for the initiation of smoking-induced cancers.

To gain insights from the pathological viewpoint, we also performed disease-focused enrichment analysis on those genes found to be significantly methylated by smoking in both blood and buccal cells (Fig. 17.3). The disease with the most significant enrichment was cancer (Fig. 17.3). This again indicates that many of these genes methylated by smoking indeed are associated with cancer.

5 Subnetwork Constructed from the 11 Common Cancer-Related Pathways

After obtaining the 11 common pathways from both blood and buccal samples, we selected 48 nonredundant genes based on their biological functions and appearance frequencies from these pathways and used them to construct a cancer-associated molecular subnetwork (Fig. 17.4). The well-documented cancer-related genes *NOTCH1*, *CDKN1A*, *EGR1*, *AKT3*, *TNF*, *MMP9*, and *SMARCA4* are located in the center of this subnetwork.

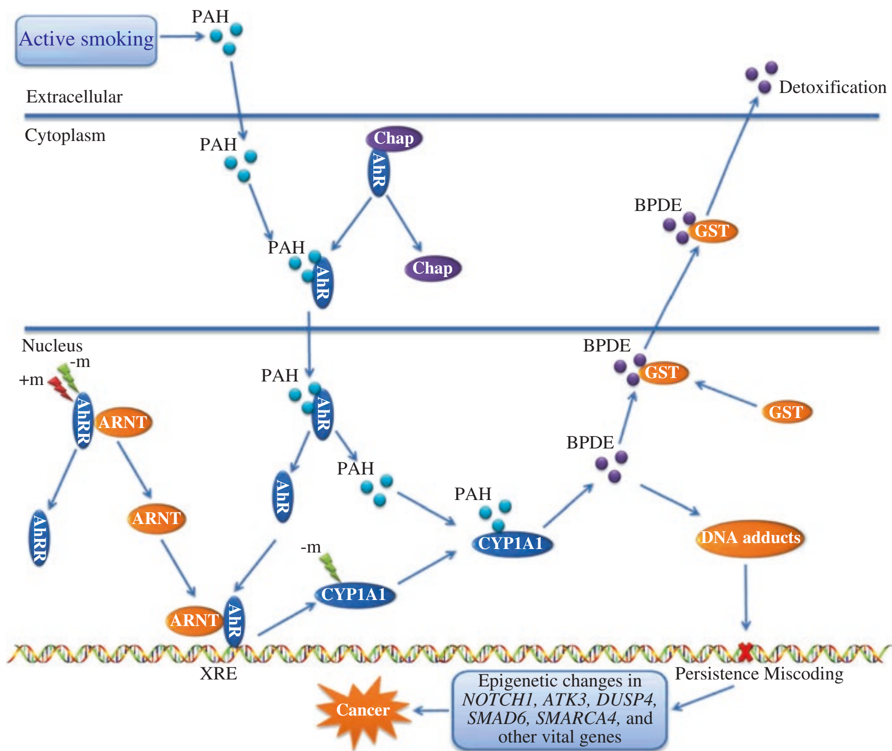

Fig. 17.1 The pathway of "aryl hydrocarbon receptor signaling"-initiated smoking-related cancer. Arrows show event flow. –m represents hypomethylation, and +m represents hypermethylation. Under normal circumstances, toxic substances from cigarette smoke, including PAHs, nitrosamines, and aromatic amines, enter the bloodstream through the alveolar capillary system and are taken up by pulmonary cells. Toxic chemicals such as the PAHs bind to transcription factor AhR, which results from the dissociation of AhR and an associated chaperone protein (Chap) complex. After translocating to the nucleus, PAHs and AhR dissociate, and AhR is dimerized with ARNT, which is produced from the AhRR–ARNT complex. The resulting complex binds to the XRE in the promoter of *CYP1A1* to enhance the expression of CYP1A1. The CYP1A1 then metabolizes PAHs into hydrophilic intermediates such as B[a]-7,8-dihydrodiol-9,10-epoxide (BPDE), which can be detoxified through the glutathione S-transferase (GST) family of enzymes or, in an alternative reaction, form DNA adducts. Under abnormal circumstances, *CYP1A1* is -m or *AhRR* has altered methylation (−m or +m) that may extraordinarily enhance the expression of CYP1A1, which could induce more DNA adduct formation that results in miscoding of the DNA sequence. Under long-term smoking exposure, the DNA sequence suffers persistent miscoding that triggers epigenetic changes in many critical cancer genes, such as *NOTCH1*, *ATK3*, *DUSP4*, *SMAD6*, and *SMARCA4*

Fig. 17.2 Schematic representation of the major enriched pathways underlying smoking-attributable cancers. Accumulating evidence indicates that smoking prominently induces cancer development. Based on the DNA methylation-enriched genes associated with smoking, we identified various overrepresented pathways. The major pathways were then linked on the basis of their biological relations originating from the database of IPA and the literature. The dashed line representing the link between two pathways was derived from published reports

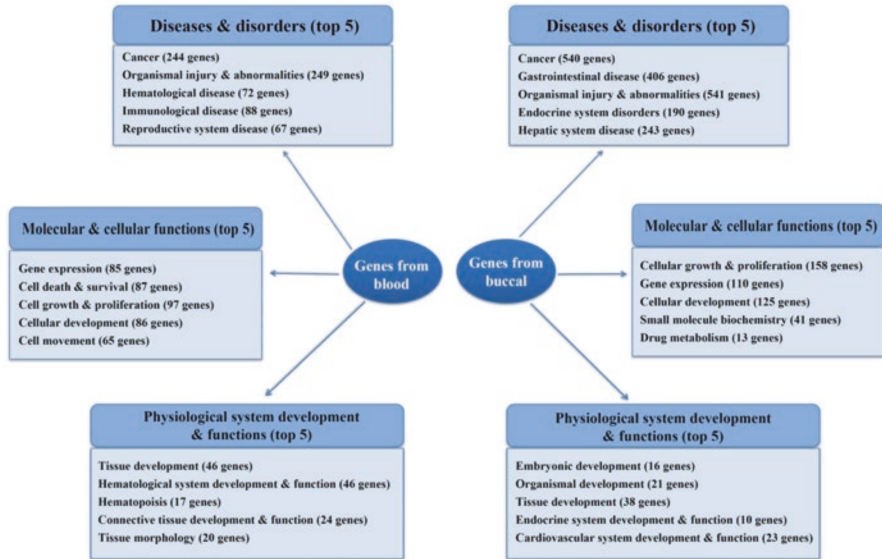

Fig. 17.3 Functional enrichment analysis of the two-hit-based genes from blood and buccal samples using IPA software. The top five terms of different categories ranked by P values are shown. The dark blue box lists the name of the functional category. The light blue box represents the specific category, and the numbers in parenthesis are the number of genes enriched in the specific category

6 48 Smoking-Related Methylated Genes Contribute to Lung Cancer

To gain further evidence of the contribution of the 48 methylated genes to cancer, we investigated the relation between RNA expression and methylation for the genes, data for which were downloaded from the large-scale database of TCGA (http://cancergenome.nih.gov/) with level 3 DNA methylation data and level 3 RNA expression data and lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) data. Among these genes, we found 148 methylation sites, with the largest number located in the gene body and 5′-untranslated region (UTR). After examining the correlation between methylation loci and RNA expression, we found that large portions of the methylation loci were significantly positively or negatively correlated with RNA expression in both LUAD (Fig. 17.5a) and LUSC (Fig. 17.5b). Most of the methylation loci correlated with RNA expression are located in the gene body and 5′-UTR in both LUAD and LUSC.

As documented in the literature, many of the 48 genes play essential roles, and have been implicated, in a variety of cancers. For example, the hub gene of *NOTCH1*, encoding one of the four Notch receptors, has an important role in a signaling pathway involved in multifaceted regulation of cell survival, proliferation, tumor angiogenesis, and metastasis (Fiuza and Arias 2007). A substantial body of research

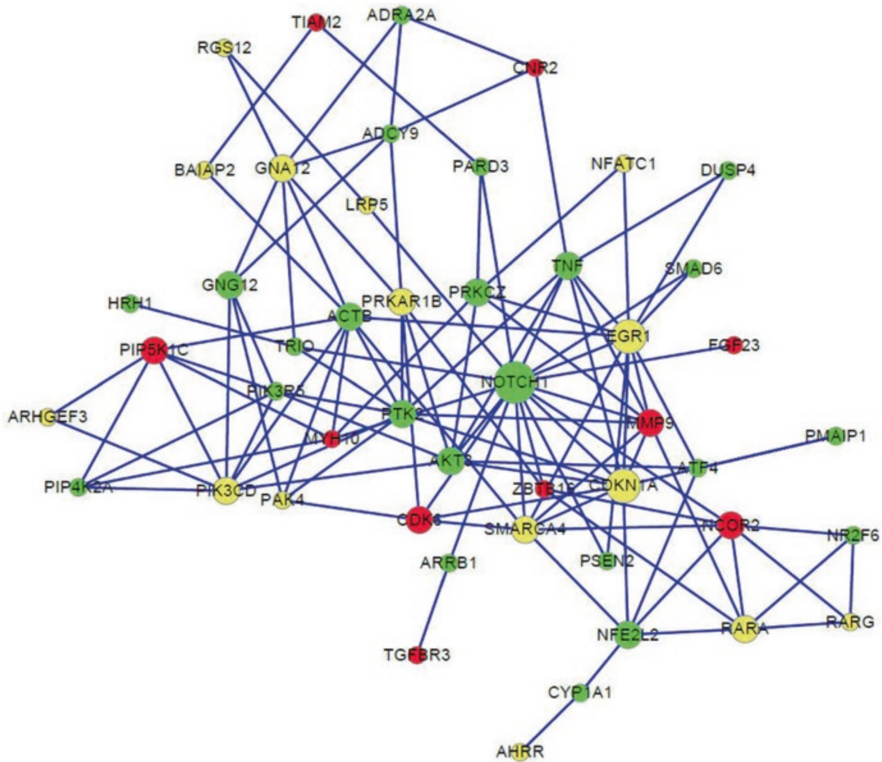

Fig. 17.4 Gene subnetwork constituted by genes from the 11 common oncogenic pathways. The protein–protein interactions shown were based on the database of STRING v 10.0. We used Cytoscape software to depict the subnetwork. The color of a node indicates the methylation direction of CpG loci in a gene: red = hypermethylation; green = hypomethylation; and yellow = hypermethylation and hypomethylation but at different sites. The edges of the genes represent predicted functional links. The number of edges in each gene was used for determining the node size, of which *NOTCH1* is the biggest

shows that *NOTCH1* correlates with the pathology of cancer (Radtke and Raj 2003). By cross talking with many other critical cancer genes and pathways, *NOTCH1* plays a fundamental role in cancer pathogenesis. Aberrant smoking-regulated methylation of *NOTCH1* thus may create a greater risk of smoking-induced cancer. Besides, the SWI/ShNF chromatin-remodeling complex, which has been linked to lung, pancreatic, breast, and colon cancer (Medina and Sanchez-Cespedes 2008), comprises a catalytic subunit of either *SMARCA4* or *SMARCA2*. The product of *SMARCA4* uses the energy from ATP hydrolysis to modify chromatin structure. Both mutation and methylation influence the expression of *SMARCA4* in cancers such as Burkitt lymphoma (Kretzmer et al. 2015), ovarian carcinoma (Jelinic et al. 2014), and lung cancer (Medina et al. 2004). Consistently, two methylated loci (cg18040892 and cg23963476) correlate inversely to a significant extent with expression of *SMARCA4* RNA in LUSC tissues. The degree of methylation of the

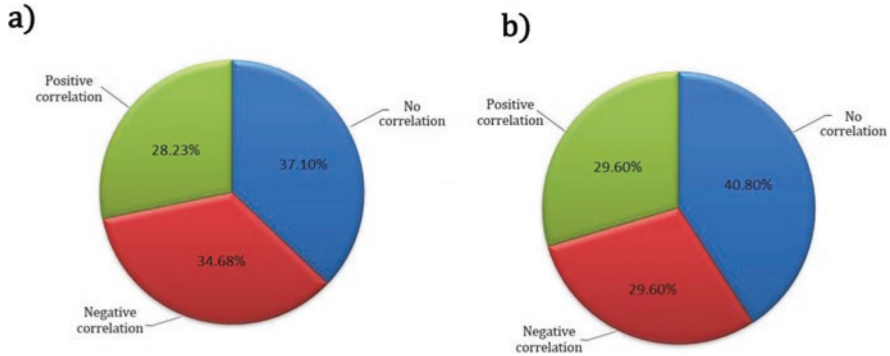

Fig. 17.5 Methylation loci of the 48 genes. Figure depicts proportion of methylation loci that showed no, positive, or negative correlation with RNA expression in LUAD (**a**) and LUSC (**b**) samples

cg23963476 probe, which is hypomethylated in smokers (Dogan et al. 2014), is significantly lower in LUSC than in control tissues, suggesting that smoking-associated hypomethylation of *SMARCA4* elicits lung cancer.

Moreover, we found that most of the methylation loci that correlated with RNA expression were differentially expressed in the control tissues vs. cancer significantly in both LUAD and LUSC samples. This is especially true for *DUSP4*. There were two methylation probes (cg07151117 and cg24379915) of this gene that show significant correlation with RNA expression in both LUAD (Fig. 17.6a, b) and LUSC. The cg07151117 probe has the strongest inverse correlation between methylation and expression in LUAD samples ($r = -0.742$; $P < 0.001$; Fig. 17.6a). The cg24379915 probe correlates negatively with *DUSP4* expression in these specimens ($r = -0.657$; $P < 0.001$; Fig. 17.6b). Compared with normal tissues, there were two hypomethylation probes of *DUSP4* in cancer tissues (Fig. 17.6c, d). Consistently, the associations of smoking with the two methylation probes of *DUSP4* in LUAD (Fig. 17.7a, b) were in line with the finding that these two CpG loci of *DUSP4* tend to be hypomethylated in smokers, as found by previous EWASs (Dogan et al. 2014; Guida et al. 2015).

The *DUSP4* gene, which interacts with the hub genes *TNF* and *EGR1*, plays an important role in the subnetwork of 48 genes linked to oncogenesis. *DUSP4*, which belongs to dual-specificity phosphatase (DUSPs) family and regulates the activity and location of MAPKs, is a negative controller of extracellular-regulated kinase activity and is upregulated in EGFR-mutant lung cancer cell lines compared with K-*ras*-mutant cells (Britson et al. 2009). Coincidently, allelic loss of *DUSP4* causes underexpression of *DUSP4* in EGFR-mutant lung adenocarcinoma (Chitale et al. 2009). In addition, *DUSP4* clearly acts as a tumor suppressor (Armes et al. 2004; Waha et al. 2010) or promotes cancer progression (Gröschl et al. 2013; Lawan et al. 2011) depending on cancer type. In the present study, we found that two smoking-associated methylation probes (cg07151117 and cg24379915) that are correlated with expression of *DUSP4* RNA are significantly hypomethylated in both LUAD

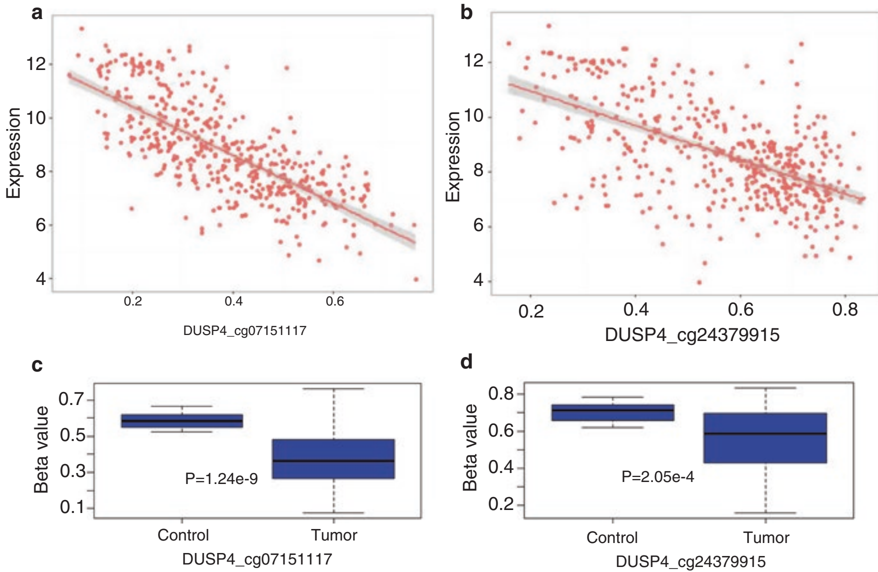

Fig. 17.6 Two methylation probes of *DUSP4* in LUAD samples. **a**, **b** Correlations of cg07151117 probe (**a**) and cg24379915 probe (**b**) with RNA expression in control and cancer cells. **c**, **d** Extent of methylation of cg07151117 probe (**c**) and cg24379915 probe (**d**) in control and cancer cells. *P* value was calculated by the Wilcoxon-rank sum test

and LUSC cancer tissues compared with the control samples. These results indicate that hypomethylated *DUSP4* is involved in smoking-induced lung cancer. Together, our proposed subnetwork of 48 genes is not only enriched for genes associated with cancer but also links those genes to smoking-attributable cancer.

7 Conclusions Remarks

In recent years, many studies have emphasized the association of smoking with DNA methylation, which is a critical mediating factor in the pathogenesis of cancer. In light of epidemiologic evidence that cigarette smoking is highly correlated with cancer, we performed a systematic bioinformatics analysis with the goal of revealing the underlying mechanism of smoking-attributable cancer from an epigenetic point of view. This study revealed a group of genes and pathways implicated in the pathology of interest. Based on the findings from the current study and previous biological evidence, we present a schematic model for elucidating the biological effects of smoking on cancer pathogenesis (Fig. 17.2).

The present study marks one of the first comprehensive pathway-based analyses of the abnormal methylation of DNA in adult smokers. Our findings demonstrate that cigarette smoking causes prominent alterations in DNA methylation that is

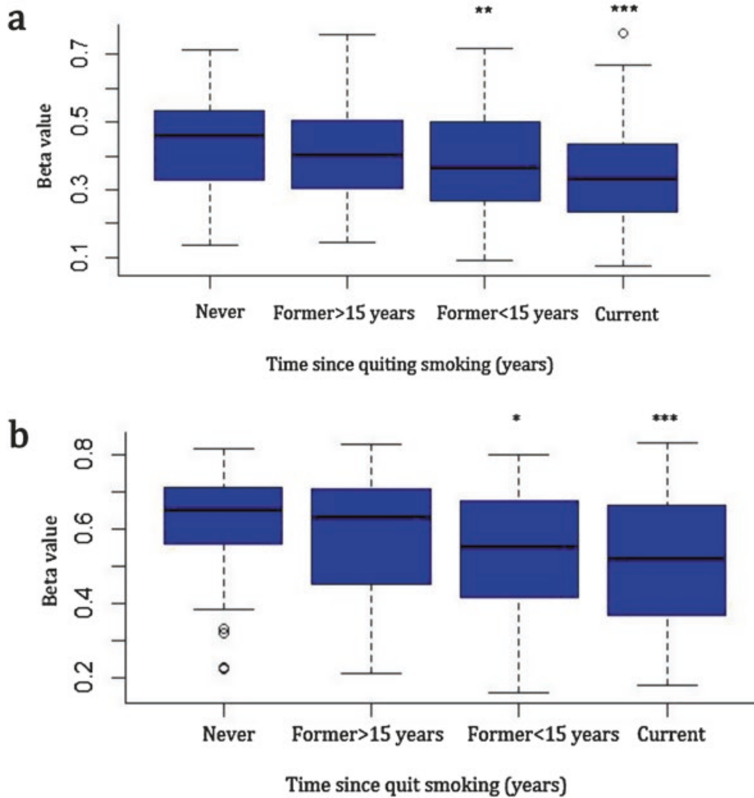

Fig. 17.7 Associations between smoking and methylation of *DUSP4* in LUAD. Methylation probes of cg07151117 (**a**) and cg24379915 (**b**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

enriched in numerous genes and pathways implicated in cancer pathology. This provides strongly and holistically epigenetics-based evidence in support of the carcinogenic effect of smoking.

However, our understanding of the contribution of smoking-related DNA methylation to cancer pathogenesis is still in an early stage. More studies are warranted to reveal the specific role(s) of particular genes aberrantly methylated in response to smoking in the development of cancer. Such understanding will have implications for the personalized treatment of smoking-attributable cancer.

Acknowledgment This chapter was modified from the published work by our group in *Scientific Report* (Ma and Li 2017). The related contents are reused with permission.

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Chapter 18
Evolutionary Relations of Genes Encoding Nicotinic Acetylcholine Receptor Subunits

Abstract Although many subunits of nAChRs have been identified in humans and other species, their evolutionary relations and biological functions are largely unknown. The primary purposes of this chapter are to explain the molecular evolutionary history and divergence times of nAChRs and to identify essential amino acid residues for the complementary component of the acetylcholine binding site. By analyzing 123 nucleotide sequences from 23 species using different bioinformatics programs, we revealed that homooligomer-forming subunits (α7–α10) diverged before the split between vertebrates and invertebrates. After this divergence, other α and non-α subunits evolved independently within each lineage but with convergence in the evolution of the subunits. In the invertebrate lineage, this gene duplication seems to have occurred not long before the group split into nematodes and insects. Furthermore, we suggest that asparagine at position 4 (N4) in loop E is essential for the complementary component of the acetylcholine binding site, which corresponds to complementary loops.

Keywords Evolution · Nicotinic acetylcholine receptors · nAChRs · Subunits · N4 loop · Alpha subunit · Non-alpha subunit · Vertebrate · Invertebrate · Phylogenetic analysis · Bootstrapping · Divergence

1 Introduction

The initial cloning and sequencing of Torpedo electric organ nicotinic acetylcholine receptor (nAChR) subunits enabled the identification of a family of diverse yet homologous genes encoding nAChR subunits in the brain and muscle of both vertebrate and invertebrate species. Numerous studies have documented that nAChRs are involved in a wide range of neuronal activities, including cognitive function and neuronal development and degeneration (Changeux et al. 1998; Picciotto and Zoli 2008).

To date, 10 α (α1–α10) and 4 β (β1–β4) subunits have been reported in vertebrates. As an example, all known nAChR subunits from humans are shown in Table 18.1. Of these, at least six α subunits (α2–α7) and three β subunits (β2–β4) are expressed in the mammalian central nervous system and govern the ionotropic cholinergic mechanism. Each nAChR is formed by five homologous subunits

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Table 18.1 Chromosomal location and characteristics of 16 human nAChR subunits

nAChR subunit	Chromosomal location	Gene size (kb)	Exons	mRNA (bp)	Protein (amino acid)
CHRNA1	2q31.1	16.64	9	1816	482
CHRNA2	8p21.2	18.51	0	2684	529
CHRNA3	15q25.1	28.24	6	2321	622
CHRNA4	20q13.33	14.75	6	2206	627
CHRNA5	15q25.1	29.71	6	3578	515
CHRNA6	8p11.21	15.93	6	2164	494
CHRNA7	15q13.2	142.25	10	6162	534
CHRNA9	4p14	19.63	5	2015	479
CHRNA10	11p15.4	5.8	5	1945	450
CHRNB1	17p13.1	12.65	11	2557	501
CHRNB2	1q21.3	12.25	6	5866	502
CHRNB3	8p11.21	39.99	6	2293	458
CHRNB4	15q25.1	17.48	6	2972	498
CHRND	2q37.1	10.48	12	2941	517
CHRNG	2q37.1	6.6	12	2187	517
CHRNE	17p13.2	5.3	12	3030	496

arranged around a central ion channel. The nAChR β3 and α5 subunits are considered structural or accessory, as they do not form functional receptors when expressed alone or in binary complexes with any other single subunit. However, they seem capable of integrating into complexes containing at least one other α and one other β subunit (Conroy and Berg 1995; Kuryatov et al. 2008). The diverse list of nAChRs includes those assembled with single α subunits (α7, α8, α9) (Couturier et al. 1990; Elgoyhen et al. 1994), multiple α subunits with (α2α5β2, α3α5β2, α3α5β4, α4α5β2) (Balestra et al. 2000; Conroy and Berg 1998; Conroy et al. 1992; Vernallis et al. 1993) or without (α7α8, α9α10) supplemental β subunits (Elgoyhen et al. 2001; Gotti et al. 1994), single α and multiple β subunits (α3β2β4, α3β3β4, α6β2β3) (Boorman et al. 2000; Colquhoun and Patrick 1997; Gotti et al. 2010; Groot-Kormelink et al. 1998), and multiple α and β subunits (α3β2β4α5, α6α4β2β3) (Gerzanich et al. 1998; Gotti et al. 2010), as well as heteromeric nAChRs formed via pairwise combinations of α2, α3, α4, α5, α6, or α7 with either the β2 or β4 subunit (Boulter et al. 1987; Deneris et al. 1988; Duvoisin et al. 1989; Goldman et al. 1987; Liu et al. 2009). Thus, the number of potential subtypes of nAChRs is large, and determining the stoichiometry of each association has been challenging in most cases (Plazas et al. 2005). Similar challenges exist in classifying the nAChR subunits as α or β and in deciphering the nAChR subtypes and their stoichiometry in invertebrates (Jones et al. 2007; Williamson et al. 2009). For example, the *Caenorhabditis elegans* genome contains the largest nAChR gene family described so far, of which 29 subunits can be predicted as nAChR subunits, and 32 subunits show the closest homology to vertebrate and invertebrate nAChR subunits, although, at present, they are designated “orphan” subunits (Jones et al. 2007). Generation and validation of predictive hypotheses or models would be of much interest to the scientific community for in silico deciphering of the combinational roles of vertebrate and invertebrate subunits.

Given the complexity of this gene family and its broad biological functions, it is of interest to understand how nAChR subunits have evolved and how they may be related to each other. Several studies (Le Novere and Changeux 1995; Ortells and Lunt 1995; Tsunoyama and Gojobori 1998) on the evolutionary history of the nAChR family indicates that the subunits can be classified into several major groups, with the first gene duplication occurring approximately 1.0–1.6 billion years ago and the last one about 400 million years ago. A number of questions remain to be addressed. For example, even though all the early studies (Le Novere and Changeux 1995; Ortells and Lunt 1995; Tsunoyama and Gojobori 1998) showed that the α7 and α8 subunits diverged first, the subsequent evolutionary processes were not defined. Although advances have been made in understanding the evolution of nAChR gene families in some model organisms or organisms of economic and medical importance (Jones et al. 2005; Shao et al. 2007), the phylogeny of the invertebrate subunits was not clearly inferred because an insufficient number of subunits were known when these studies were conducted. Therefore, a more comprehensive study using the most recent sequence information is necessary to better delineate the evolutionary relations among the nAChR subunits.

Neurotransmitter binding sites of different subunits are composed of a principal component in loops A, B, and C and a complementary component in loops D, E, and F (Corringer et al. 2000). Although the presence of two consecutive cysteines in loop C was suggested to be essential for the characterization of the principal component (Galzi and Changeux 1995), it is unclear whether there are any essential amino acid residues in the complementary component. Therefore, the second objective of this study was to identify essential amino acid residues for the complementary component, if they exist, on the basis of our multiple sequence alignments. Furthermore, we propose a more generalized quaternary organization model for nAChRs based on a specific quaternary organization of the muscle-type, homo-oligomeric α7, and hetero-oligomeric α4β2 receptors (Corringer et al. 2000). In spite of the extensive experimental studies conducted on nAChR subunits, the combinational roles of vertebrate α5 and β3 and most invertebrate subunits have not yet been deciphered completely.

2 Information on 123 nAChR Subunit Sequences from Different Species Used to Derive Their Evolutionary Relations

All sequences were extracted from DDBJ/EMBL/GenBank using keyword searches. All predicted nAChR subunit sequences from *C. elegans* and *D. melanogaster*, and redundant sequences for each subunit in GenBank, were excluded. After these filtrations, 123 subunit sequences, representing 23 species, were used in the evolutionary analysis reported here. A detailed list of names, abbreviations, accession numbers, and references for these subunits is given in Table 18.2.

Table 18.2 Genes used in this study. Gene abbreviations are those used in the text and tree

Gene abbreviation	Species	Accession number
Asu-α	*Ascaris suum*	AJ011382
Bta-α1	*Bos taurus*	X02509
Bta-α3	*Bos taurus*	X57032
Bta-α7	*Bos taurus*	X93604
Bta-β1	*Bos taurus*	X00962
Bta-δ	*Bos taurus*	X02473
Bta-ε	*Bos taurus*	X02597
Bta-γ	*Bos taurus*	M28307
Cau-α3	*Carassius auratus*	X54051
Cau-β2	*Carassius auratus*	X54052
Cau-nα2	*Carassius auratus*	X14786
Cau-nα3	*Carassius auratus*	M29529
Cel-deg3	*C. elegans*	U19747
Cel-ce21	*C. elegans*	X83887
Cel-acr3	*C. elegans*	Y08637
Cel-ce13	*C. elegans*	X83888
Cel-lev	*C. elegans*	X98601
Cel-acr2	*C. elegans*	X86403
Cel-acr4	*C. elegans*	AF077307
Cel-unc38	*C. elegans*	X98600
Cfa-α1	*Canis familiaris*	AB021708
Dme-sad	*D. melanogaster*	X52274
Dme-α3	*D. melanogaster*	Y15593
Dme-α4	*D. melanogaster*	AJ272159
Dme-als	*D. melanogaster*	X07194
Dme-rel	*D. melanogaster*	M20316
Dme-sbd	*D. melanogaster*	X55676
Dme-β3	*D. melanogaster*	AJ318761
Dre-α1	*Danio rerio*	U70438
Gga-α1	*Gallus gallus*	AJ250359
Gga-α10	*Gallus gallus*	AJ295624
Gga-α2	*Gallus gallus*	X07339
Gga-α3	*Gallus gallus*	M37336
Gga-α4	*Gallus gallus*	X07348
Gga-α5	*Gallus gallus*	J05642
Gga-α6	*Gallus gallus*	X83889
Gga-α7	*Gallus gallus*	X52295
Gga-α8	*Gallus gallus*	X52296
Gga-α9	*Gallus gallus*	AF082192
Gga-β2	*Gallus gallus*	X53092
Gga-β3	*Gallus gallus*	X83739
Gga-β4	*Gallus gallus*	J05643

(continued)

Table 18.2 (continued)

Gene abbreviation	Species	Accession number
Gga-δ	*Gallus gallus*	K02903
Gga-γ	*Gallus gallus*	K02904
Hco-hcal	*Haemonchus contortus*	U72490
Hsa-α1	*Homo sapiens*	Y00762
Hsa-α2	*Homo sapiens*	U62431
Hsa-α3	*Homo sapiens*	Y08418
Hsa-α4	*Homo sapiens*	X89741
Hsa-α5	*Homo sapiens*	Y08419
Hsa-α6	*Homo sapiens*	U62435
Hsa-α7	*Homo sapiens*	X70297
Hsa-α9	*Homo sapiens*	AJ243342
Hsa-α10	*Homo sapiens*	AF199235
Hsa-β1	*Homo sapiens*	X14830
Hsa-β2	*Homo sapiens*	X53179
Hsa-β3	*Homo sapiens*	Y08417
Hsa-β4	*Homo sapiens*	Y08416
Hsa-δ	*Homo sapiens*	X55019
Hsa-ε	*Homo sapiens*	X66403
Hsa-γ	*Homo sapiens*	X01715
Hvi-α1	*Heliothis virescens*	AJ000399
Hvi-α2	*Heliothis virescens*	AF096878
Hvi-α3	*Heliothis virescens*	AF096879
Hvi-α7-1	*Heliothis virescens*	AF143846
Hvi-α7-2	*Heliothis virescens*	AF143847
Hvi-β1	*Heliothis virescens*	AF096880
Lmi-α1	*Locusta migratoria*	AJ000390
Lmi-α2	*Locusta migratoria*	AJ000391
Lmi-α3	*Locusta migratoria*	AJ000392
Lmi-β	*Locusta migratoria*	AJ000393
Mmu-α1	*Mus musculus*	X03986
Mmu-α4	*Mus musculus*	AF225912
Mmu-α5	*Mus musculus*	AF204689
Mmu-α6	*Mus musculus*	AJ245706
Mmu-α7	*Mus musculus*	L37663
Mmu-β1	*Mus musculus*	M14537
Mmu-β2	*Mus musculus*	AF145286
Mmu-δ	*Mus musculus*	L10076
Mmu-ε	*Mus musculus*	X55718
Mmu-γ	*Mus musculus*	M30514
Mmu-ht	*Mus musculus*	M74425
Mpe-α1	*Myzus persicae*	X81887
Mpe-α2	*Myzus persicae*	X81888

(continued)

Table 18.2 (continued)

Gene abbreviation	Species	Accession number
Mpe-α3	*Myzus persicae*	AJ236786
Mpe-α4	*Myzus persicae*	AJ236787
Mpe-α5	*Myzus persicae*	AJ236788
Mse-als	*Manduca sexta*	Y09795
Ovo-nα	*Onchocerca volvulus*	L20465
Rno-α	*Rattus norvegicus*	M15682
Rno-α1	*Rattus norvegicus*	X74832
Rno-α10	*Rattus norvegicus*	AF196344
Rno-α2	*Rattus norvegicus*	M20292
Rno-α3	*Rattus norvegicus*	L31621
Rno-α4	*Rattus norvegicus*	L31620
Rno-α5	*Rattus norvegicus*	NM_017078
Rno-α6	*Rattus norvegicus*	L08227
Rno-α7	*Rattus norvegicus*	L31619
Rno-β1	*Rattus norvegicus*	NM_012528
Rno-β2	*Rattus norvegicus*	L31622
Rno-β3	*Rattus norvegicus*	J04636
Rno-β4	*Rattus norvegicus*	J05232
Rno-δ	*Rattus norvegicus*	X74835
Rno-ε	*Rattus norvegicus*	X13252
Rno-γ	*Rattus norvegicus*	X74834
Rno-mls	*Rattus norvegicus*	X15834
Rra-α1	*Rattus rattus*	X74832
Rra-α3	*Rattus rattus*	L31621
Rra-α9	*Rattus rattus*	U12336
Rra-β1	*Rattus rattus*	X74833
Rra-β2	*Rattus rattus*	L31622
Rra-δ	*Rattus rattus*	X74835
Rra-γ	*Rattus rattus*	X74834
Sgr-αl1	*Schistocerca gregaria*	X55439
Tca-α1	*Torpedo californica*	J00963
Tca-β1	*Torpedo californica*	J00964
Tca-δ	*Torpedo californica*	J00965
Tca-γ	*Torpedo californica*	J00966
Tco-tar1	*Trichostrongylus colubriformis*	U56903
Tma-α1	*Torpedo marmorata*	M25893
Xla-α1	*Xenopus laevis*	X07067
Xla-α1a	*Xenopus laevis*	X17244
Xla-β1	*Xenopus laevis*	U04618
Xla-δ	*Xenopus laevis*	X07069
Xla-ε	*Xenopus laevis*	U19612
Xla-γ	*Xenopus laevis*	X07068

The first letter represents the genus, and the second two letters represent the species, followed by the name of the subunit

2.1 Evolutionary Relations of the nAChR Family

Of the three programs, i.e., PILEUP of GCG package, CLUSTAL W, and SAM-T99, used to conduct multiple sequence alignments, the alignments resulting from SAM-T99 analysis, which performed best, consisted of the conserved Hidden Markov Model (HMM) sites as well as non-conserved insertion sites. The non-conserved sites were excluded from the analysis because they are less informative in phylogenetic analysis. The resulting amino acid sequence alignments were used as a template for the proper alignment of the corresponding DNA sequences. For both the amino acid and nucleotide sequence alignments, columns containing one or more nucleotide or amino acid deletions among all members were deleted to generate an alignment profile for the best conserved sites. The third nucleotide position of each codon of the aligned HMM and of the most conserved sites was deleted to produce nucleotide sequence alignments for the first and second codon positions.

Then, we used three phylogenetic analysis methods, namely, the neighbor-joining (NJ) method of CLUSTAL W (Sievers et al. 2011), the maximum parsimony (MP) method of GCG, and the maximum likelihood (ML) method of PHYLIP 3.69 (http://evolution.genetics.washington.edu/phylip.html) to construct phylogenetic trees. Six alignments (two for amino acid sequences, HMM sites and most conserved sites; four for nucleotide sequences, HMM sites and most conserved sites for the first and second codon positions) were used for each method. The robustness of the phylogenetic hypotheses was tested by bootstrapping. All bootstrap analyses of DNA and amino acid sequences for the MP and NJ methods involved 1000 replications of the original alignments. For all analyses, the serotonin-gated ion channel receptor subunit (Mmu-5HT) was used as an out-group to root the trees. The combinational roles of unverified subunits were predicted on the basis of the assumption that the subunits that belong to the same group within a phylogenetic tree frequently have similar functional roles.

Most of the phylogenetic trees constructed by different phylogenetic analysis methods showed that the 123 sequences included in the study are classified into six major groups (Fig. 18.1). Groups I (containing both vertebrate and invertebrate subunits), *Heliothis virescens* (an insect) and *C. elegans*, and II (vertebrate subunits only) diverged earlier from a common ancestor than did groups III–VI. All other invertebrate subunits were classified into group III or IV, whereas the other remaining vertebrate subunits were assigned to either group V or group VI. Groups III, IV, V, and VI were further divided into two or four subgroups, as noted. Regardless of the analytical method used, members classified in groups I–VI were almost the same, even though the topology among a few subgroups may have differed. The bootstrap values of subunits appearing in each major group from 1000 bootstrap replicates were 95%, 100%, 100%, 99%, 79%, and 86% for groups I to VI, respectively. In Table 18.3, we summarize the subunits classified into each group and the corresponding subgroups.

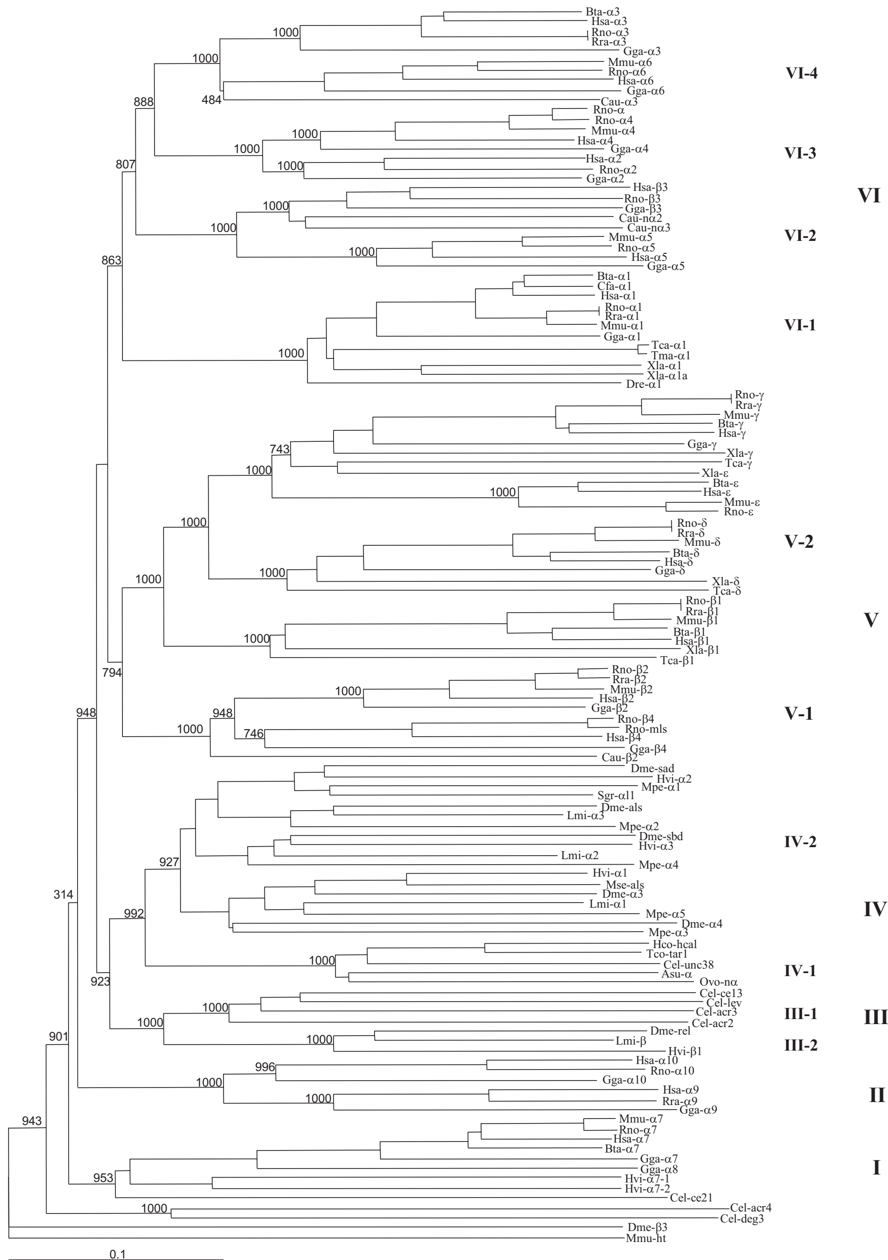

Fig. 18.1 Phylogenetic tree of 123 nAChR subunits representing 23 species (12 vertebrates and 11 invertebrates) with the serotonin receptor as an out-group. The tree was constructed by the ML method using nucleotide HMM site alignments. According to their phylogeny and biological functions, these subunits were classified into six major groups

Table 18.3 Summary of subunits classified in major groups and subgroups according to the phylogenetic trees

Group	Subgroup	Subunits
I		Vertebrate α7, α8 subunits
		Invertebrate Cel-ce21, Hvi-α7-1, and Hvi-α7-2 subunits
II		Vertebrate α9, α10 subunits
III	III-1	Nematoda β subunits
	III-2	Insecta β subunits
IV	IV-1	Nematoda α and Ovo-nα subunits
	IV-2	Insecta α and Dme-sbd subunits
V	V-1	Vertebrate β2 and β4 subunits
	V-2	Vertebrate non-α subunits
VI	VI-1	Vertebrate α1 subunits
	VI-2	Vertebrate α5 and β3 subunits
	VI-3	Vertebrate α2 and α4 subunits
	VI-4	Vertebrate α3 and α6 subunits

Group I, a direct descendant of the ancestral gene, includes both vertebrate (α7 and α8) and invertebrate (Hvi-α7-1, Hvi-α7-2, and Cel-ce21) subunits. Following the evolution of group I, group II diverged to generate the α9 and α10 subunits. The third split generated two clusters, groups III–IV and V–VI. Groups III and IV are composed of all the remaining invertebrate subunits, in which group IV (α subunits, Dme-sbd, and Ovo-nα) diverged from group III (non-α subunits). Subgroups III-2 and IV-2 are composed solely of insect subunits, whereas subgroups III-1 and IV-1 contain nematode subunits only. On the other hand, groups V and VI are composed of vertebrate subunits only, in which group V (non-α subunits) has diverged from group VI (α and β3 subunits). In group V, subgroup V-1 (β2 and β4) diverged from subgroup V-2 (vertebrate muscle non-α subunits). In group VI, subgroups VI-1 (α2, α4) and VI-2 (α3, α6) diverged successively before a further split between subgroups VI-3 (α5, β3) and VI-4 (muscle α).

2.2 *Evolutionary History of the nAChR Family*

The phylogenetic tree indicates that, prior to the invertebrate/vertebrate transition, two duplications of the ancestor of the nAChR subunits occurred, with the first event yielding group I and the second yielding group II and the ancestor gene for groups III–VI. Following separation of the vertebrates from the invertebrates, the ancestor gene for groups III–VI produced groups V and VI in vertebrates and groups III and IV in invertebrates, which was inferred to occur before nematodes split from insects. Based on these phylogenic analyses, an evolutionary model of this gene family is presented in Fig. 18.2.

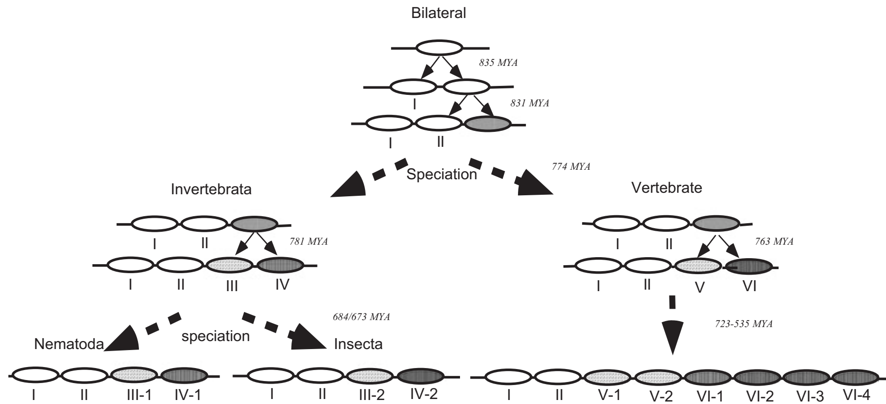

Fig. 18.2 Proposed model for the evolution of the nicotine acetylcholine receptor family. There were two gene duplications, which produced groups I and II, before the split between vertebrates and invertebrates. In vertebrates, one duplication produced the division between insects and nematodes

These phylogenetic analyses showed that, prior to the invertebrate/vertebrate transition, two duplications of the ancestor gene for the nAChR family occurred, with the first event yielding group I and the second producing group II and the ancestor gene for groups III–VI (see Figs. 18.1 and 18.2). After separation of vertebrates from invertebrates, the ancestor gene for groups III–VI produced groups V (non-α subunits) and VI (α subunits) in vertebrates and groups III (non-α subunits) and IV (α subunits) in invertebrates, which occurred before nematodes split from insects. This independent generation of α and non-α subunits in vertebrates and invertebrates suggests a convergence in the evolution of nAChR subunits. We also found that N4 of loop E may be essential for the complementary component of acetylcholine binding sites.

2.3 Times of Divergence for the nAChR Family

To obtain an evolutionary age for this gene family, we estimated the time of divergence among the family's major groups by calculating intergroup average *p* distances with the MEGA2 package (Kumar et al. 1994). Because the *p* distance is not proportional to the evolutionary time, the Poisson-corrected distance (d) was used to estimate the time of divergence among the major groups. The relation between *p*- and Poisson-corrected distances is $d = -ln(1\text{-}p)$ (Nei 1987).

From the fossil records, it was estimated that the chicken diverged from mammals approximately 310 million years ago (Benton 1990). The distances were initially calculated for the α1-7, α9-10, β2-4, δ, and γ subunits between chickens and mammals and then used to calculate the ratio of distance to evolutionary time.

Table 18.4 Times of divergence for the major groups and subgroups of nAChR subunits

Group/subgroup	Average Kimura's distance	Time of divergence (10^6 years)
I cf. II–VI	0.918	835
II cf. III–VI	0.9141	831
III–IV cf. V–VI	0.8513	774
III cf. IV	0.8588	781
V cf. VI	0.8389	763
III-1 cf. III-2	0.7524	684
IV-1 cf. IV-2	0.7401	673
V-1 cf. V-2	0.7958	723
VI-1 cf. VI 2–4	0.7018	638
VI-2 cf. VI 3–4	0.6729	612
VI-3 cf. VI-4	0.588	535

Assuming diverged time: Chicken/mammals 310 MYA (Benton 1990)

Relative to this ratio, we obtained the divergence time for the major duplication events of this family. As shown in Table 18.4, our results indicate that three major gene duplications took place around 1.1–1.5 billion years ago, which is consistent with previous reports (Le Novere and Changeux 1995; Ortells and Lunt 1995).

3 Identification of Putative Essential Amino Acid Residues for Complementary Binding Sites

All vertebrate subunits except α5, α10, and β3 were divided into two groups, with one group having the complementary component of the binding site while the other group does not. Based on the multiple sequence alignments, loops D, E, and F were examined in detail to identify the amino acid residues conserved in the complementary component but not in the noncomplementary component. These residues were assumed to be essential for the complementary component.

On the basis of their contribution to neurotransmitter binding sites, vertebrate subunits can be divided into four functional groups: (1) principal-complementary subunits (PC subunits; i.e., α7–9), which contribute to both the principal and the complementary components; (2) principal subunits (P subunits; i.e., α1–4 and α6), which contribute to the principal but not to the complementary component; (3) complementary subunits (C subunits; i.e., β2, β4, δ, and ε), which contribute to the complementary but not to the principal component; and (4) structural subunit (S subunit; i.e., β1), which contributes to neither the principal nor the complementary component. By examining the amino acid sequence alignments within loops D, E, and F, we found that asparagine at position 4 (N4) in loop E is conserved in both C (i.e., vertebrate δ, ε, β2, and β4) and PC (vertebrate α7–9) subunits but is not conserved in S (β1) or P (vertebrate α1–4, α6) subunits (Fig. 18.3a). This suggests that N4 in loop E is essential for the complementary component of these subunits.

```
(A)
PC-subunits

            loop   loop      loop                  loop           loop                     loop
           | D  | |  A  | |   E                 | |  B  | |       F                 | |      C      |
Bta-α7     WLQMTW WKPDILLY FHTNVL...VNS.....SGHCQYL WSYGGWSL QE.......................ADI KRSEKFY ECCKE...P...YP
Gga-α7     WLQMYW WKPDILLY FHTNVL...VNS.....SGHCQYL WTYGGWSL QE.......................ADI KRTESFY ECCKE...P...YP
Hsa-α7     WLQMSW WKPDILLY FHTNVL...VNS.....SGHCQYL WSYGGWSL QE.......................ADI KRSERFY ECCKE...P...YP
Rno-α7     WLQMSW WKPDILLY FHTNVL...VNA.....SGHCQYL WSYGGWSL QE.......................ADI KRNEKFY ECCKE...P...YP
Mmu-α7     WLQMSW WKPDILLY FHTNVL...VNA.....SGHCQYL WSYGGWSL QE.......................ADI KRNEKFY ECCKE...P...YP
Gga-α8     WLQMYW WVPDILLY FHTNVL...VNY.....SGSCQYI WTHSGWLI -e.......................ADI KRNELYY ECCKE...P...YP
Gga-α9     WIRQSW WRPDIVLY VNTNVV...LRY.....DGKITWD WTYNGNQV DS.......................GDL VKNVITY GCCSE...P...YP
Hsa-α9     WIRQIW WRPDIVLY VNTNVV...LRY.....DGLITWD WTYNGNQV DS.......................GDL VKNVISY GCCSE...P...YP
Rra-α9     WIRQTW WRPDIVLY VNTNVV...LRY.....DGLITWD WTYNGNQV DS.......................GDL VKNVISY GCCSE...P...YP

P-subunits
Rra-α1     RLKQQW WRPDVVLY KFTKVL...LDY.....TGHITWT WTYDGSVV DQ.......................PDL WKHWVFY SCCPNt P...YL
Bta-α1     RLKQQW WRPDLVLY KFTKVL...LDY.....TGHITWT WTYDGSVV DQ.......................PDL WKHWVFY ACCPSt P...YL
Cfa-α1     RLKQQW WRPDLVLY KFTKVL...LDY.....TGHITWT WTYDGSVV DQ.......................PDL WKHWVFY ACCPSt P...YL
Dre-α1     RLKQQW WKPDLVLY HETKVL...LEH.....TGMITWT WTYDGNLV DR.......................PDL WKHWVYY ACCPDt P...YL
Gga-α1     RLKQQW WRPDLVLY KYTKVL...LEH.....TGKITWT WTYDGTMV DR.......................PDL WKHWVYY ACCPDt P...YL
Hsa-α1     RLKQQW WRPDLVLY KFTKVL...LQY.....TGHITWT WTYDGSVV DQ.......................PDL WKHSVTY SCCPDt P...YL
Rno-α1     RLKQQW WRPDVVLY KFTKVL...LDY.....TGHITWT WTYDGSVV DQ.......................PDL WKHWVFY SCCPNt P...YL
Xla-α1     RLKQQW WSPDLVLY KDTKIL...LEY.....TGKITWT WTYDGSLL DR.......................PDL WKHWVYY TCCPDk P...YL
Xla-α1a    RLKQQW WRPDIVLY QETKVL...LDY.....TGKIIWL WTYDGTLV DR.......................PDL WKHWVYY DCCPEt P...YL
Tca-α1     RLRQQW WLPDLVLY HMTKLL...LDY.....TGKIMWT WTYDGTKV DR.......................PDL WKHWVYY TCCPDt P...YL
Mmu-α1     RLKQQW WRPDVVLY KFTKVL...LDY.....TGHITWT WTYDGSVV DQ.......................PDL WKHWVFY SCCPTt P...YL
Tma-α1     RLRQQW WLPDLVLY HMTKLL...LDY.....TGKIMWT WTYDGTKV DR.......................PDL WKHWVYY TCCPDt P...YL
Gga-α2     WLKQEW WIPDIVLY HMTKAH...LFS.....NGKVKWV WTYDKAKI HH.......................VDL RYNSKKY DCCTE...I...YP
Hsa-α2     WLKQEW WIPDIVLY HMTKAH...LFS.....TGTVHWV WTYDKAKI QT.......................VDL TYNSKKY DCCAE...I...YP
Rno-α2     WLKQEW WIPDIVLY HMTKAH...LFF.....TGTVHWV WTYDKAKI RT.......................VDL TYNSKKY DCCAE...I...YP
Bta-α3     WLKQIW WKPDIVLY DKTKAL...LKY.....TGEVTWI WSYDKAKI SS.......................MNL YKHDIKY NCCEE...I...YP
Cau-α3     WLRHIW WRPDIVLY DKTKAL...LKY.....DGTITWV WTYDKAKI SK.......................VNL YKHDIKY NCCEE...I...YP
Gga-α3     WLKHIW WKPDIVLY DKTKAL...LKY.....TGDVTWI WSYDKAKI ST.......................MNL YKHDIKY NCCEE...I...YT
Hsa-α3     WLKQIW WKPDIVLY DKTKAL...LKY.....TGEVTWI WSYDKAKI SS.......................MNL YKHDIKY NCCEE...I...YP
Rno-α3     WLKQIW WKPDIVLY DKTKAL...LKY.....TGEVTWI WSYDKAKI SS.......................MNL YKHEIKY NCCEE...I...YQ
Gga-α4     WVKQEW WRPDIVLY HLTKAH...LFY.....DGRIKWM WTYDKAKI SH.......................VDQ NYNSKKY ECCTE...I...YP
Hsa-α4     WVKQEW WRPDIVLY HLTKAH...LFH.....DGRVQWT WTYDKAKI SR.......................VDQ TYNTRKY ECCAE...I...YP
Mmu-α4     WVKQEW WRPDIVLY HLTKAH...LFY.....DGRVQWT WTYDKAKI SR.......................VDQ TYNTRKY ECCAE...I...YP
Rno-α4     WVKQEW WRPDIVLY HLTKAH...LFY.....DGRVQWT WTYDKAKI SR.......................VDQ TYNTRKY ECCAE...I...YP
Rno-α      WVKQEW WRPDIVLY HLTKAH...LFY.....DGRVQWT WTYDKAKI SR.......................VDQ TYNTRKY ECCAE...I...YP
Gga-α5     WLKQEW WIPDIVLY -STKTV...VKY.....DGTIAWT WTYDGSQV YE.......................VDK SKGNRTD GCC--...W...YP
Hsa-α5     WLKQEW WTPDIVLF -STKTV...IRY.....NGTVTWT WTYDGSQV QD.......................VDK SKGNRTD SCC--...W...YP
Mmu-α5     WLKQEW WIPDIVLF -STKTV...VRY.....NGTVTWT WTYDGSQV QD.......................VDR SKGNRTD SCC--...W...YP
Rno-α5     WLKQEW WIPDIVLF -STKTV...VRY.....NGTVTWT WTYDGSQV QD.......................VDR SKGNRTD SCC--...W...YP
Gga-α6     WLRHIW WKPDIVLY GKTKAL...LRY.....DGMITWT WTYDKAKI SK.......................VDM YKHDIKY NCCEE...I...YT
Hsa-α6     WLRHIW WKPDIVLY GKTKAL...LKY.....NGMITWT WTYDKAEI SK.......................VDM YKHDIKY NCCEE...I...YT
Mmu-α6     WLRHIW WKPDIVLY GKTKAL...LKY.....DGVITWT WTYDKAEI SK.......................VDM YKHDIKY NCCEE...I...YT
Rno-α6     WLRHVW WKPDIVLY GKTKAL...LKY.....DGVITWT WTYDKAEI SK.......................VDM YKHDIKY NCCEE...I...YT

S-subunits
Bta-β1     YLDLEW WLPDVVLL LDINVV...VSS.....DGSMRWQ YSYDSSEV QExqevy...................IHE RLIQPSV DPRGGqeqR...RE
Hsa-β1     YLDLEW WLPDVVLL LDISVV...VSS.....DGSVRWQ YSYDSSEV PDqqghqeih................IHE RLIQPPG DPRGGreqQ...RQ
Mmu-β1     YLDLEW WLPDVVLL LDINVV...VSF.....EGSVRWQ YSYDSSEV EExqevy...................IHE RLIQLPG DQRGG...KeghHE
Rno-β1     YLDLEW WLPDVVLL LDINVV...VSF.....EGSVRWQ YSYDSSEV DPdqqexqeiy...............IHE RLIHLPG DRRGG...KeghRE
Rra-β1     YLDLEW WLPDVVLL LDINVV...VSF.....EGSVRWQ YSYDSSEV DPdqqexqeiy...............IHE RLIHLPG DRRGG...KeghRE
Xla-β1     YLEMAW WTPDIVLM LQVDVL...VSP.....NGNVTWH YTYGADEV DAnqkevtqay...............IFP RKNSSPN DPL--...-...YE
Tca-β1     FLNLAW WQPDIVLM LHVNVL...VQH.....TGAVSWQ YTYDTSEV DAkqerevkeiy..............INK RKNWRSD DPS--...-...YE

C-subunits
Cau-β2     WLTQEW WLPDIVLY FYCNAV...VSN.....TGDIFWL WTYDRTEL DF.......................ASR RKNEDPN DLT--...-...YL
Gga-β2     WLTQEW WLPDVVLY FYSNAV...ISY.....DGSIFWL WTYDRTEI EV.......................ASL RRNENPD DST--...-...YV
Hsa-β2     WLTQEW WLPDVVLY FYSNAV...VSY.....DGSIFWL WTYDRTEI EV.......................ASL RRNENPD DST--...-...YV
Mmu-β2     WLTQEW WLPDVVLY FYSNAV...VSY.....DGSIFWL WTYDRTEI DV.......................ASL RRNENPD DST--...-...YV
Rno-β2     WLTQEW WLPDVVLY FYSNAV...VSY.....DGSIFWL WTYDRTEI DV.......................ASL RRNENPD DST--...-...YV
Rra-β2     WLTQEW WLPDVVLY FYSNAV...VSY.....DGSIFWL WTYDRTEI DV.......................ASL RRNENPD DST--...-...YV
Gga-β4     WLNQEW WLPDIVLY LYTNAI...VQN.....NGSIRWL WTYDHTEI SM.......................ASM RRTENPL DPN--...-...YV
Hsa-β4     WLKQEW WLPDIVLY VYTNLI...VRS.....NGSVLWL WTYDHTEI PT.......................ASM RRTVNPQ DPS--...-...YV
Rno-β4     WLKQEW WLPDIVLY VYTNVI...VRS.....NGSIQWL WTYDHTEI PT.......................AIM RRTVNPQ DPS--...-...YV
Rno-mls    WLKQEW WLPDIVLY VYTNVI...VRS.....NGSIQWL WTYDHTEI PT.......................AIM RRTVNPQ DPS--...-...YV
Bta-δ      WIEQGW WLPEIVLE YSCNVL...IYP.....SGSVYWL LKYTTKEI AEedgrsypvewii...........IDP RVNVDPS VPLDS...Pn RQ
Gga-δ      WVEQSW WLPEIVLE YYCNVL...VYN.....TGYVYWL LAYNAQEI ESdpeteknyxvewii.........IDP RKNIHPS..YPTESs..E...HQ
Mmu-δ      WIDHAW WLPEIVLE YACNVL...VYD.....SGYVTWL LKYTAKEI EEennrsypiewii...........IDP KLNVDPS VPMDSt N...HQ
Hsa-δ      WIEHGW WLPEIVLE YSCNVL...VYH.....YGFVYWL LKYTAKEI DAkenxtypvewii...........IDP RVNVDPR APLDS...Ps RQ
Rno-δ      WIDHAW WLPEIVLE YACNVL...VSD.....SGHVTWL LKYTAKEI EEednrsypiewii...........IDP KVNVDPS VPMDSt N...HQ
Xla-δ      WVELAW WQPQLILE YYSNVL...ISS.....DGFMYWL LTYNAKEI DLdeasgrvypvewii.........IDP KKNIDRS LSPESt K...YQ
Rra-δ      WIDHAW WLPEIVLE YACNVL...VSD.....SGHVTWL LKYTAKEI EEednrsypiewii...........IDP KVNVDPS VPMDSt N...HQ
Tca-δ      WMDHAW WIPDIVLQ YFCNVL...VRP.....NGYVTWL LNYDANEI DTidgkdypiewii...........IDP KKNIYPD KFPNGt N...YQ
Bta-ε      WIGIDW WLPEIVLE YEANVL...VSE.....GGYLSWL QTYNAEEV DDeqktiskid..............IDT VIRRHDG..DSAGG...Pq..ET
Mmu-ε      WIGIDW WLPEIVLE YDSNVL...VYE.....GGYVSWL QTYNAEEV DDdqntinkid..............IDT MIRRYEG GSTEG...Pq ET
Hsa-ε      WIGIDW WLPEIVLE YDANVL...VYE.....GGSVTWL QTYNAEEV DNdqktinkid..............IDT VIRRHHG GATDG...Pq ET
Xla-ε      WVQIAW WLPDIVLE YYANVL...VYN.....TGYIYWL KTYSANEI DDetqlpfdqvd.............IDR RKILNPK YSKEDl R...YQ
Rno-ε      WIGIEW WLPEIVLE YDCNVL...VYE.....GGSVSWL QTYNAEEV DDdqnainkid..............IDT MIRHYEG GSTED...Pq ET
Bta-γ      WIEMQW WRPDIVLE LYCNVL...VSP.....DGCVYWL QTYSTNEI EDqqtiewif...............IDP KMLLDEA APAEEa G...HQ
Gga-γ      WIEMQW WLPDIVLE LYTNVL...VYP.....DGSIYWL QTYSANEI EEqqtiewif...............IDP RKIINSG RETPDdi Q...YQ
Hsa-γ      WIEMQW WRPDIVLE LYCNVL...VSP.....DGCIYWL QTYSTNEI EDqqtiewif...............IDP KMLLDPA APAQEa G...HQ
Mmu-γ      WIEMQW WRPDIVLE LYCNVL...VSP.....DGCIYWL QTYSTSEI EDqqaiewif...............IDP KMLLDSV APAEEa G...HQ
Rno-γ      WIEMQW WQPDIVLG LYCNVL...VSP.....DGCIYWL QTYSTSEI EDqqaiewif...............IDP KMLLDPV TPAEEa G...HQ
Rra-γ      WIEMQW WQPDIVLG LYCNVL...VSP.....DGCIYWL QTYSTSEI EDqqaiewif...............IDP KMLLDPV TPAEEa G...HQ
Tca-γ      WIEIQW WLPDVVLE YYANVL...VYN.....DGSMYWL QTYNAMEV EEqeavewih...............IDP KKNYNWQ LTKDDt D...FQ
Xla-γ      WVEMQW WLPDVGLE LYTNTL...VSS.....DGSMYWL QTYSANEI DEqtiewie................IDP KRIINHR LPRDDv N...YQ
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Fig. 18.3 Multiple amino acid sequence alignments of neurotransmitter binding loops. (**a**) Sequence alignments of the vertebrate subunits whose combinational roles have been verified by experiments. The N4 of loop E is conserved in all complementary components but not in non-complementary components. (**b**) Sequence alignments of the subunits whose combinational roles were predicted. The combinational roles were inferred from the hypothesis proposed in this chapter (see text for details)

Fig. 18.3 (continued)

On the basis of essential amino acid residues for acetylcholine binding sites and the more general quaternary model of nAChRs proposed herein, a hypothesis is proposed that infers a combinational role of nAChR subunits according to amino acid sequence. From this hypothesis, we predict that vertebrate α10 and invertebrate Hvi-α7 and Cel-α1 subunits represent the PC subunits, and subunits in group IV and vertebrate α5 represent the P subunits, with the exceptions of Ovo-nα and Dme-sbd. Subunits in group III are predicted to represent C subunits, whereas vertebrate β3, Ovo-nα, and Dme-sbd represent S subunits (Fig. 18.3b). Surprisingly, these predictions are consistent with our inference from phylogenetic analysis that subunits belonging to group I/II can form homo-oligomers, and subunits of group III need those of group IV in order to form hetero-oligomers. Experiments demonstrated that co-expression of α5 with other α and β subunits in oocytes reduces the binding affinity of expressed nAChRs (Ramirez-Latorre et al. 1996; Wang et al. 1996; Yu and Role 1998), whereas co-expression of β3 does not (Groot-Kormelink et al. 1998). These findings support our hypothesis that β3 is an S subunit, whereas α5 is a P subunit. Previously, it was reported that α5 is not a P subunit because it does not possess the conserved tyrosine in loop C, as is found in other α subunits (Wang et al. 1996). However, this idea is not consistent with our prediction. In addition, if the α5 subunit had not been a principal subunit, the C9C10 would have been lost, as were

other C and S subunits. Because of the lack of a conserved tyrosine residue, we suggest that α5's binding property may differ from that of the other α subunits. As the α5 subunit cannot form a functional nAChR with any β subunit, it is likely that any nAChR containing a α5 subunit has two kinds of P subunits (e.g., α2α5β2, α3α5β2, α3α5β4, α4α5β2, etc.) However, it is worth noting that Fucile et al. (1997) showed that the human nAChR α5 subunit forms a functional receptor along with human nAChR β2 or β4 subunits. Hence, it may not be too long before we know that the nAChR α5 subunit in fact is a P subunit.

4 Evolutionary Relations Among Different nAChR Subunits of Vertebrate and Invertebrate Species

On the basis of the results illustrated in Fig. 18.2, we expect that subunits in groups I and II would be present in both vertebrates and invertebrates because they were generated prior to the vertebrate/invertebrate transition. However, examination of the subunits indicates this is so for group II but is questionable for group I for invertebrates. This may result from other unidentified invertebrate subunits that may fall in group I. In fact, this expectation has gained support from the work reported by Szczupak et al. (1998), which showed that there exist receptors in the leech that possess a pharmacological profile similar to that of the nAChR α9 subunit. Although the biological functions of vertebrate α10, invertebrate Hvi-α7-1, and the Hvi-α7-2 subunits are unknown, we suspect they are capable of forming functional homo-oligomers. This prediction is based on reports that other members of groups I/II, such as vertebrate α7–9 and Cel-ce21, can form functional homo-oligomers (Couturier et al. 1990; Elgoyhen et al. 1994; Gerzanich et al. 1994; Schoepfer et al. 1990). Although we predict vertebrate α10 to be a homopentamer, experimental results do not show formation of any detectable homomoric (rat) α10 nAChR either *in vitro* or in rat cochlear and vestibular hair cells (Elgoyhen et al. 2001). Rather, the α10 subunit partners with the α9 subunit in vitro to form functional receptors, indicating that α10 may be acting as a "structural subunit" (Elgoyhen et al. 2001). Further, the authors of the same paper argue that most likely, such a receptor (α9α10) exists in vivo, as is inferred from indirect experimental results. It is probable that future experimental work will show that there exists homopentameric α10–nAChR in vivo but not an α9α10–nAChR, validating our phylogenetic classification and predictive hypothesis. Duplication events leading to the emergence of groups I and II and the ancestral gene for the remaining subunits preceded the split between vertebrates and invertebrates. Subsequent gene duplications occurred independently in vertebrates and invertebrates. In vertebrates, a gene duplication has led to the generation of the ancestral forms of groups VI (α) and V (non-α) subunits. Interestingly, co-expression of a member of subgroup V-1 and a member of subgroups VI-1 or -2 is a prerequisite for the formation of an acetylcholine-gated ion channel with the accepted stoichiometry of 2α3β (Anand et al. 1991; Cooper et al. 1991). Similarly, co-expression of subgroups V-2 and VI- 1 is functionally necessary for the

vertebrate muscle nAChR (Machold et al. 1995). This finding suggests that the ancestor of group V or VI cannot become a functional nAChR unless it is combined with another subunit.

A similar evolutionary process took place in invertebrates. Following the split between vertebrates and invertebrates, the ancestors of groups IV (α subunits) and III (non-α subunits) emerged via gene duplication, which occurred not long before the split of nematodes and insects. On the basis of the genetic distances between these two subgroup members, we suspect that this gene duplication occurred about 1.2 million years ago. Further gene duplication seems to have occurred independently in nematodes and insects. Most experimental data from invertebrates suggest that the α subunit in group IV needs the β subunit in group III to form a functional receptor (Bertrand et al. 1994; Fleming et al. 1997; Huang et al. 1999, 2000; Lansdell et al. 1997). However, several research groups (Amar et al. 1995; Gundelfinger and Hess 1992; Sawruk et al. 1990a; Sgard et al. 1998) have documented that Sgr-αL1, Mpe-α1, Mpe-α2, and Dme-ard from group IV-2 can form homo-oligomers (in a heterologous expression system), albeit only at high concentrations of an agonist (in the mM range). Inward currents generated by these homo-oligomers are minute (in the nAmp range), implying that they may not be physiologically functional homo-oligomers (Gundelfinger and Hess 1992; Sawruk et al. 1990a, b). This indicates that they require a partner β subunit from group III-2 to form a functional receptor. Similarly, an *Ascaris suum* α subunit (GenBank No. AJ011382) from group IV has been confirmed by Williamson et al. (2009) to be a true α subunit by independent cloning (GenBank EU053155) and functional expression studies.

Cel-deg3 (encoding an nAChR subunit, which, in the region of transmembrane domain II, is most similar to the neuronal α7 subunits from rat and chicken) from *C. elegans* cannot be assigned to any of the six major groups in our trees. Treinin and Chalfie (1995) showed that deg-3 and des-2 are functionally dependent acetylcholine subunits from *C. elegans*: they co-assemble to form a functional receptor. In an analysis by Jones et al. (2007), deg-3 groups with other nAChR subunits (including des-2) from *C. elegans* do not fall clearly into an α or β category. In fact, we found that several subunits cannot be classified, as is shown by Jones et al. (2007), into any of the six major groups according to the predicted nicotinic receptor subunits from genome sequences of *C. elegans* and *D. melanogaster*. Although some of these subunits are predicted to be acetylcholine receptor subunits, they show the greatest similarity to members of the *cys*-loop ligand-gated ion channel (LGIC) superfamily, which also includes γ-aminobutyric acid (GABA), serotonin (5-HT), glycine, glutamate, and histamine receptors and chloride-gated channels (Dent 2006). Hence, most of these subunits were not included in our analysis. We expect that more groups or subgroups of nAChR subunits will be identified in the near future as more genomic sequences from human, mouse, rat, and other species become available. The assignment of α or β category to some of these subunits has not been easy because there is a possibility that some subunits co-assemble with other proteins (ancillary proteins involved in assembly and trafficking of the receptors) to form functional receptors. This point may be underscored by the finding by Boulin et al.

(2008) that eight genes are required for functional reconstitution of the *C. elegans* levamisole-sensitive acetylcholine receptor.

5 Proposed Hypothesis for the Evolution of nAChR Subunit Family

The abovementioned results lead to the following hypothesis for the evolution of the nAChR family. The common ancestor of nAChRs functioned essentially as a homo-oligomer in the primitive Bilateria, which had both C9C10 in loop C and N4 in loop E. Before the split between vertebrates and invertebrates, the diversity of the nAChR family was enhanced by generation of different homo-oligomers. This initial increase in diversity might have had little evolutionary space to gain a variety of pharmacological properties because of great structural pressure caused by an "all-in-one" configuration of a homo-oligomer subunit. In a homo-oligomer, one subunit is responsible for both binding and allosteric transition. The evolutionary split between α and non-α subunits, taking place independently in both vertebrates and invertebrates, enabled the presence of principal and complementary binding sites on different subunits. Decoupling of the principal and complementary binding sites might have provided the much-needed evolutionary space, which may be the driving force for the convergent evolution of α and non-α subunits. According to our hypothesis on the combinational role, these subunits might be S subunits, as they lack both the C9C10 of loop C and the N4 of loop E. The S subunit in a pentameric receptor would give even broader evolutionary space to generate more diverse pharmacological properties in nAChRs. For example, the sophisticated stoichiometry of the vertebrate muscle receptor (α1β1δγ) might evolve under the pressure of fast signal transduction in neuromuscular junctions, which might give a better chance for survival. Two complementary subunits and one structural subunit may give the muscle receptor appropriate features for adaptation of fast signal transduction. In the nervous system, α5 and β3 may enable more complexity of the stoichiometry of neuronal nAChR, which may be as sophisticated as muscle nAChRs, to fit the more advanced functional requirements that accompany evolution. In short, the evolutionary history of the nAChR family confirms that living organisms would tend to use every possible way to generate more complex derivatives from their original templates to meet the challenges brought about by changes in environmental factors.

6 Concluding Remarks

This chapter shows that, prior to the invertebrate/vertebrate transition, two duplications of the ancestor gene for nAChR subunits occurred, with the first yielding group I subunits and the second producing group II subunits and the ancestor gene

for groups III–VI. After separation of vertebrates from invertebrates, the ancestor gene for groups III–VI produced, independently, groups V and VI in vertebrates and groups III and IV in invertebrates, which occurred before nematodes split from insects. Our phylogenetic analyses further demonstrate that nAChRs evolved from a simple homo-oligomer to complex hetero-oligomers, with the same strategy being used in both vertebrates and invertebrates. In a hetero-oligomer, different subunits are dedicated to different functional roles; i.e., principal binding, complementary binding, and allosteric transition. This would make vast pharmacological varieties available, which is considered a prerequisite for complex neuronal activities. Finally, according to multiple sequence comparisons, we found that the conserved N4 of loop E may be essential for the complementary binding component. A hypothesis on the prediction of nAChRs' combinational role has been proposed on the basis of both the essential amino acid residues of the acetylcholine binding sites and a more general quaternary organization model for PC, P, C, and S subunits. According to this hypothesis, the combinational roles of invertebrate receptors and some vertebrate receptors are predicted.

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Chapter 19
Management, Pharmacotherapies, and Precision Medicine for Smoking Cessation

Abstract Although 70% of smokers want to quit, only 4–7% are successful in the long term. Cigarette smoking persists because of both the addictive propensity of nicotine and the low success rates of existing treatments. Smoking cessation outcomes are influenced by both smoking cessation medications and genetic factors which include variations in the enzymes that metabolize nicotine and in nAChR subunit genes that are the primary targets of nicotine and of treatment medications. Identifying subjects with appropriate variants is an essential element in improving smoking cessation outcomes. In this chapter, we highlight recent progress in our understanding of how genetic variants in the pharmacological targets of nicotine and smoking cessation medications could be used to tailor cessation therapy and increase the success rate.

Keywords Smoking dependence · Consulting · Smoking cessation · Quitting · DSM-V · Genetic markers · Fagerström Test for Nicotine Dependence · FTND · Intervention · 5As · Biomarkers · SNPs · *Bupropion hydrochloride* · *Zyban* · *Varenicline tartrate* · Chantix · Nicotine replacement therapy · *CYP2A6* · *CYP2B6* · Tobacco dependence · *CHRNA5/A3/B4*

1 Clinical Diagnostic Criteria for Nicotine Dependence (ND)

The DSM-V (APA 1994) defines tobacco use disorder as a problematic pattern of tobacco use leading to clinically significant impairment or distress, as manifested by at least two of the following criteria during a 12-month period: (1) using tobacco in larger amounts or for a longer period than intended; (2) a persistent desire or unsuccessful effort to reduce or control tobacco use; (3) a great deal of time spent in activities necessary to obtain or use tobacco; (4) craving or a strong desire or urge to use tobacco; (5) recurrent tobacco use resulting in a failure to fulfill obligations of major roles at work, school, or home; (6) continued tobacco use despite persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of tobacco; (7) important social, occupational, or recreational activities given up or reduced because of tobacco use; (8) recurrent tobacco use in situations in which it is physically hazardous; (9) tobacco use being continued despite knowledge of

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Table 19.1 Fagerström Test for Nicotine Dependence (FTND)

Question	Selections	Score
1. How many cigarettes a day do you usually smoke?	1–10	0
	11–20	1
	21–30	2
	31 or more	3
2. How soon after you wake up do you smoke your first cigarette?	Within 5 min	3
	6–30 min	2
	31–60 min	1
	More than 60 min	0
3. Do you smoke more frequently during the first 2 h of the day than during the rest of the day?	Yes	1
	No	0
4. Which cigarette would you most hate the most to give up?	The first cigarette in the morning	1
	Any other cigarette	0
5. Do you find it difficult to refrain from smoking in places where it is forbidden, such as church, at the movies, etc.?	Yes	1
	No	0
6. Do you still smoke even when you are so ill that you are in bed most of the day?	Yes	1
	No	0
Total		0–10

having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by tobacco; (10) tolerance; and (11) withdrawal symptoms.

There are some concerns about the DSM criteria for ND, one of the primary ones being that they do not measure the degree of dependence. Thus, other instruments have been used as a supplement or replacement in both clinical and research settings. The Fagerström Test for Nicotine Dependence (FTND) is one of the most popular questionnaires to characterize the degree to which the patient is physically dependent on cigarette smoking (Heatherton et al. 1991) (Table 19.1).

Another questionnaire for measuring ND is the Wisconsin Inventory of Smoking Dependence Motives (WISDM) (Piper et al. 2004), which provides greater information regarding various domains of smoking motivation. This is a relatively new scale but with accumulating evidence suggesting its utility. Other scales are used less frequently and tend to be restricted to research applications. However, only limited direct research has evaluated the appropriateness of these new criteria.

2 Clinical Characteristics of ND

Patterns of tobacco use differ considerably in different users. Individuals may consume tobacco sporadically or daily, in small to large amounts, and in single vs. multiple forms. The degree to which an individual's pattern of tobacco use is

elicited by exposure to tobacco stimuli (e.g., others smoking, distressing circumstances) is variable, as is the type, intensity, and duration of the particular withdrawal symptoms experienced. The general characteristics of the tobacco-using population are changing, with higher prevalence rates now evident among those of lower socioeconomic status and educational attainment and those with psychiatric diagnoses or symptoms. Overall, the risk of relapse post-cessation is high, particularly for those who quit without professional assistance or among certain subgroups (e.g., pregnant women). Patients who have used tobacco products for an extended period often present with signs of compromised health, generally related to the length of their smoking history. Depressive or anxiety symptoms or both are relatively common, and the clinician should be aware that the suicide rate for smokers is substantially higher than that for the general population; the risk for former smokers falls in between.

3 Genetic Screening for ND

Although there is substantial evidence for the involvement of genetic factors in ND and smoking cessation, no major Mendelian genes or variants have been identified. Familial clustering is common but not universal. Nevertheless, many variants have been implicated in ND and its treatment. Further validation is greatly needed in order to use this genetic information clinically.

Currently, there is a lack of clear and robust evidence supporting genetic testing for ND or ability to quit smoking. Even though some markers appear to be promising, most of those tests have not yet been validated clinically. Although many SNPs in various candidate genes have been associated with ND or smoking cessation, only a few of them have been replicated in multiple independent samples, and most of these findings await further replication. So far, the most convincing example is variants in the nicotinic receptor subunit *CHRNA5/A3/B4* gene cluster on chromosome 15 (see Chap. 5); a risk variant rs1051730 in this cluster has been used to predict the potential genetic risk for ND in individuals of European origin. However, the SNPs that have been investigated account for a very small proportion of the variance (e.g., <5%), and thus, their predictive clinical validity would be low.

4 Management and Pharmacotherapies for ND

Healthcare-based interventions for tobacco dependence that include both counseling and pharmacologic evidence-based components are the most effective treatment approach. In general, interventions that incorporate higher levels of effort/resources increase the likelihood of success. With respect to counseling, this means longer treatment times/more sessions, as well as input from providers from a variety of

fields. When considering medications, recent evidence indicates that higher doses (as appropriate), some combination regimens, longer duration of therapy, and initiating prior to quit date (for nicotine replacement, similar to accepted protocols for other medications) generally are associated with higher success rates.

4.1 Psychosocial Interventions

Counseling for ND greatly improves long-term success. Although intervention can be delivered in many forms, emphasis on two factors appears to be key: practical skills/problem solving and intra-treatment support. The delivery of services can differ with respect to intensity and modality. "Intensity" refers to the amount of time/number and spacing of sessions. The depth to which issues are dealt with and the extent of patient participation are likely correlated features. "Modality" is the manner in which treatment is delivered. The following is a brief overview of current options.

(a) *Brief interventions in the primary care setting*: Primary care physicians and their staffs operate in an environment that offers many advantages regarding the delivery of a standardized, brief intervention. The use of health information by experts to motivate patients, along with the capacity to counsel and provide prescription medications, is a highly effective combination. The major barriers are the limited time available to deliver these services, as well as inadequate reimbursement.

Brief interventions can be developed in light of the usual operations in the outpatient clinic. They require little money or staff time, with interventions as short as 3 min substantially increasing cessation rates. One widely employed option is the "5As" method, which involves the following: (1) asking about tobacco status at each visit, (2) advising all tobacco users to quit, (3) assessing the patient's willingness to quit, (4) assisting the patient in quitting, and (5) arranging for follow-up contact. Finally, if the intervention is unsuccessful or the provider believes a more potent intervention is necessary, patients can be referred to more intensive counseling programs.

(b) *Specialty tobacco clinic interventions*: This treatment option represents the highest end of the intensity dimension. Such programs generally are delivered by individuals who have received Tobacco Treatment Specialist training to conduct a multi-session, face-to-face program (group or individual based) that addresses numerous issues related to achieving cessation. These programs often employ aggressive pharmacotherapy and more sophisticated counseling techniques and provide extended follow-up services.

(c) *Quitelines*: Telephone-based services have the advantages of broad reach and somewhat more sophisticated counseling services, as well as easy access and relative anonymity. Pharmacotherapy options tend to be more limited, relying on patients working with their own physicians for complex options, and thus are not as

tailored or closely monitored as specialty clinic options. Overall, they may be considered of moderate intensity and represent a good option for many patients.

(d) *Other options*: Recently, other delivery modalities have emerged, including web- and cellular telephone text-based options. Although available data are limited, early findings suggest the utility of these treatments.

4.2 Approved Treatment for ND

National guidelines recommend that pharmacologic therapy be considered for all smokers attempting to quit unless it is medically contraindicated. The US Food and Drug Administration (FDA)-approved pharmacologic interventions are nicotine replacement therapies (NRTs), bupropion hydrochloride, and varenicline tartrate, all of which enjoy extensive published support for their effectiveness. Behavioral counseling is an important adjunct to any pharmacologic intervention for smoking cessation (Faessel et al. 2010).

A large proportion of the ability to quit smoking is heritable, with an estimated heritability of 50–60% (Broms et al. 2006). During recent years, genetic variation in the major pathway of nicotine metabolism has been shown to alter the quantity of cigarettes smoked. Genetic variation in nicotine metabolism and receptor genes, and in the dopaminergic pathway genes, has been implicated in the ability to quit smoking.

(a) *Nicotine replacement therapies* deliver nicotine to ease withdrawal and craving while allowing the smoker to break the behavioral habits associated with tobacco use. Withdrawal symptoms are experienced by many when attempting to quit and can reduce the likelihood of achieving and maintaining abstinence.

There are five FDA-approved nicotine replacement options. Nicotine polacrilex gum and lozenges are available in 2 mg and 4 mg doses and are sold without a prescription. Patients must be instructed as to their proper use (e.g., “chew and park” routine for the gum, avoiding ingestion of anything that alters the oral pH). The primary advantage of these products is the ability to adjust administration as needed for changing circumstances. Nicotine transdermal patches (also over-the-counter) have the advantage of maintaining steady blood nicotine concentrations over the course of the day. Both the nicotine nasal spray and the inhaler require a prescription. All NRTs may produce side effects, although these often can be reduced or eliminated with proper use tailored to the individual. The usual duration of use is approximately 3 months, although evidence is accumulating of better long-term abstinence with longer use.

(b) *Bupropion* hydrochloride (Zyban) is an atypical antidepressant with noradrenergic and dopaminergic effects. Mechanisms of action include inhibition of dopamine reuptake in the nucleus accumbens, as well as nicotine antagonism in the ventral tegmental area. The recommended and maximum dose for smoking cessation is 300 mg per day, usually taken as 150 mg twice daily. Dosing should begin at 150 mg a day, the drug being given daily for the first 3–7 days, followed by an

increase to the recommended 300 mg a day as tolerated. Typically, therapy is begun 1–2 weeks before the patient's determined smoking quit date. Treatment should continue for at least the recommended 12 weeks.

(c) *Varenicline* tartrate (Chantix) was approved by the FDA in 2006 for the treatment of ND. This medication appears to function as a partial agonist at α4β2 and full agonist at α7 nicotinic cholinergic receptors that binds with high affinity to these receptors. Nicotine stimulation of this particular receptor, with which varenicline binds with high specificity, is associated with significant mesolimbic dopamine release, which reinforces nicotine use. The high affinity with which varenicline binds to this receptor, in conjunction with its long half-life, reduces nicotine's capacity to stimulate the receptor, thereby attenuating the reinforcing properties of nicotine ingestion while providing sufficient stimulation to counter withdrawal symptoms. Dosing is up-titrated from 0.5 mg per day to 2 mg per day over the first week; 3–6 months of use is recommended.

5 Precision Medicine for ND

Almost all approaches to human genetic studies have been used to search for susceptibility genomic regions and genes for ND, including genome-wide linkage analysis and candidate gene-based or genome-wide association (GWA) studies. Although there is a great variability in the detected linkage peaks among studies, primarily because of the small samples, variations in measures of smoking behavior, and differences in ethnic backgrounds and environmental factors, genetic variants in the following three groups have received much attention:

(a) *Nicotine metabolism genes*: One of the most investigated genes of this type is *CYP2A6*, which encodes the enzyme cytochrome P450 CYP2A6. In humans, about 70–80% of nicotine is converted to cotinine by this liver enzyme, with minor contribution from CYP2B6. Nicotine is metabolized to minor compounds by additional enzymes, including FMO3 and UGT2B10. The majority of cotinine undergoes further metabolism to 3′-hydroxycotinine (3HC) in a reaction mediated exclusively by CYP2A6 (Fig. 19.1). The 3′-hydroxycotinine/cotinine ratio, known as the nicotine metabolite ratio (NMR), is an established and validated phenotypic indicator of CYP2A6 activity in daily smokers; faster CYP2A6 activity is reflected by a higher NMR (Allenby et al. 2016). Approximately 33–40% of cotinine is converted to its primary metabolite, 3HC, also by *CYP2A*6.

The *CYP2A6* gene is highly polymorphic, with many variants altering the protein's function. Variants in *CYP2A6* have been associated with several smoking-related phenotypes. Individuals can be genotyped for these variants and grouped into CYP2A6 activity groups (e.g., faster and slower metabolizers) on the basis of the predicted metabolic impact of their *CYP2A6* genotype on nicotine clearance (Benowitz et al. 2006). Smokers with reduced- or null-activity *CYP2A6* alleles (i.e., *CYP2A6*9*, *CYP2A6*12*, *CYP2A6*2*, or *CYP2A6*4*) smoke fewer cigarettes and

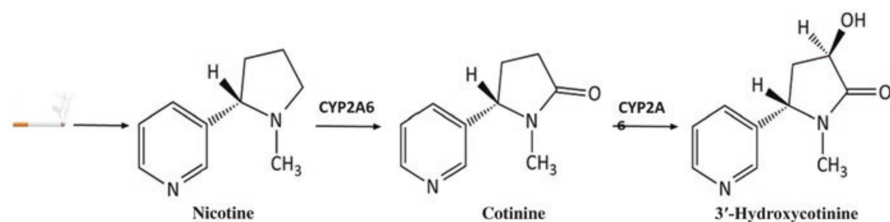

Fig. 19.1 Metabolism of nicotine to cotinine and *trans*-3′-hydroxycotinine

tend to be less nicotine dependent and more likely to quit than smokers with normal or increased enzyme activity. Several smoking cessation studies have reproducibly indicated that the nicotine metabolism rate predicts quitting success.

Given the large number of *CYP2A6* alleles, as well as the influence of environmental factors on nicotine metabolism, a phenotypic biomarker of CYP2A6 activity (3HC/cotinine) appears to be a more robust predictor of cessation than genotype. Similar to genotype-based activity groupings, smokers can be dichotomized as faster or slower metabolizers on the basis of NMR. However, there currently is no single optimized NMR cut-point to distinguish slower from faster metabolizer for cessation optimization. Different investigators have selected NMT cut-points based on sensitivity and specificity analyses of smoking cessation outcomes. Slower nicotine metabolizers have lower cigarette consumption, dependence, nAChR availability, and brain response to smoking cues than do faster nicotine metabolizers. Slower nicotine metabolizers also display higher smoking cessation rates in the absence of pharmacotherapy.

In smokers randomized to treatment based on NMR, varenicline was more efficacious than were nicotine patches in faster metabolizers but showed no or little difference in value for slower metabolizers, suggesting that varenicline is more suitable for faster metabolizers, whereas the patch is more suitable for slower metabolizers (Lerman et al. 2015).

Like *CYP2A6*, *CYP2B6* is highly polymorphic. The CYP2B6 protein is expressed in the liver and in extrahepatic tissues, including the brain, and degrades bupropion to its pharmacologically active metabolite hydroxybupropion (Kharasch et al. 2008). Thus, variation in CYP2B6 activity that alters the hydroxybupropion concentration could influence the success of bupropion-assisted smoking cessation. The common CYP2B6*6 haplotype (e.g., about 25% in Caucasians) comprises the VYP2B6*4 (rs2279343) and CYP2B6*9 (rs3745274) non-synonymous variants and is associated with lower hepatic CYP2B6 protein expression and reduced metabolism of bupropion.

In addition to its role in bupropion metabolism, CYP2B6 is believed to act in the central metabolism of nicotine. In rats, the selective inhibition of brain CYP2B, which is thought to mimic genetically slow CYP2B6 metabolism in humans, is associated with high brain nicotine concentrations and a need for a greater number of sessions to extinguish nicotine self-administrative behavior. In placebo-treated Caucasian heavy smokers, those with one or two copies of *CYP2B6*6* had lower

end-of-treatment quit rates than those with no copies of the variant (Lee et al. 2007). Thus, slow CYP2B6 activity may be associated with a higher relapse risk in subjects receiving placebo and possibly those taking bupropion.

(b) *Nicotinic receptor subunit genes*: Variation in nAChR genes, particularly in the *CHRNA5/A3/B4* cluster, located on chromosome 15q25, has been examined for association with smoking cessation success either in the absence of treatment or with active pharmacotherapy. Although variants in the *CHRNA5/A3/B4* cluster are robustly associated with small differences in cigarette consumption and ND (see Chap. 5), the association between these variants and smoking cessation outcomes has differed in various studies.

Of the SNPs in this cluster that have been investigated, rs16969968, located in *CHRNA5*, has been studied most frequently. In a meta-analysis of 24 studies in nontreatment-seeking Caucasian smokers, those with the AA genotype of rs16969968 quit a median of 4 years later than smokers with the GG genotype (Chen et al. 2015b). In a separate analysis of community-based Caucasian smokers, a high-risk haplotype, defined by rs16969968 (A allele) and rs680244 (C allele), delayed self-reported smoking cessation by a median of 2 years compared with lower-risk groups (Chen et al. 2012). In contrast, rs16969968 does not appear to be associated with quitting with the aid of pharmacotherapy. A meta-analysis in smokers receiving NRT showed no associations between rs16969968 or rs1051730 and end-of-treatment or 6-month quit rate (Leung et al. 2015). Furthermore, in several studies of treatment-seeking individuals, including those using the nicotine patch, bupropion, or varenicline, variant rs16969968 showed no association with smoking cessation or the end-of-treatment quit rate (Chen et al. 2015a; Tyndale et al. 2015). Two other SNP tagging loci, rs588765 and rs578776, in the *CHRNA5/A3/B4* cluster that have been robustly associated with minor influences on cigarette consumption and dependence in Caucasians, likewise, were not associated with cessation outcomes (Tyndale et al. 2015). Taken together, the lack of replicated findings for nAChR gene variants and smoking cessation outcomes reduces the likelihood that this genomic region will be useful in precision medicine action for smoking cessation.

Beyond the *CHRNA5/A3/B4* cluster, variation in other nAChR subunit genes also has been associated with smoking cessation. The A allele of rs2072661 in *CHRNB2* was associated with lower quitting rates in both bupropion- and placebo-treated smokers (Conti et al. 2008). Analyses in three separate placebo-controlled clinical trials revealed additional SNPs, rs3811450 and rs4292956 in *CHRNB2*, rs3787138 and rs2236196 in *CHRNA*4, and rs6494214 in *CHRNA7*, which influenced abstinence in response to varenicline (King et al. 2012). Whether these findings will be replicated in other varenicline-treated smokers or extended to other treatments remains to be determined.

(c) *Dopamine and other relevant neurotransmitter systems*: Variation in the genes involved in the dopamine system has been investigated as a potential source of differences in smoking cessation outcomes. In general, functional polymorphisms that lead to reduced dopaminergic activity are thought to contribute lower smoking cessation success (David et al. 2008).

Although the VNTR polymorphism in exon 3 of *DRD4* was not associated with overall abstinence in Caucasian smokers receiving placebo or bupropion, bupropion increased the cessation rate in smokers with one or more copies of the long allele (seven or more repeats) and showed no benefit in smokers with two copies of the short allele (fewer than seven copies) compared with placebo-treated controls (Simpson et al. 2010). In a separate study, Bergen et al. (2013) observed a larger, albeit nonsignificant, benefit of bupropion in those with the long allele than in those homozygous for the short allele. Together, these data suggest that bupropion is a more suitable treatment for smokers with the long allele of exon 3 of *DRD4*.

Genetic variants in the dopamine transporter (*SLC6A3*) and *DRD*2 genes also have been examined as potential modulators of smoking cessation outcomes. At the end of treatment, neither the 3′-VNTR polymorphisms in *SLC6A3* nor the *Taq*1A2 RFLP polymorphism located in about 10 kb of the 3′ end of *DRD2* was found to be associated with abstinence in Caucasian smokers randomized to receive either placebo or bupropion (David et al. 2007). However, the *Taq*1A2 polymorphism was associated with bupropion-assisted quitting at 6-month follow-up. In those with the *DRD2 Taq*1A2/A2 genotype, quit rates were higher for subjects taking bupropion than those on placebo. By contrast, bupropion was not associated with a greater cessation rate in *Taq*1A1 individuals. These findings highlight the potential importance of assessing multiple genes and gene–gene interactions, as opposed to single genes, to identify subgroups of smokers who are more likely to benefit from a certain treatment.

6 Concluding Remarks

The recent and current genetic studies of ND and smoking cessation have already provided a wealth of knowledge regarding the etiology of ND and its successful treatment. Insights into the molecular mechanisms underlying ND and other smoking-related behaviors should provide new biological targets for developing novel treatment drugs. Although none of the susceptibility variants is a definitive genetic screening tool for the diagnosis of ND and its treatment, many susceptibility variants have been suggested. For example, smokers with faster rates of nicotine metabolism, as determined by the NMR, have higher quit rates when taking varenicline than with the nicotine patch. On the other hand, for the slower metabolizers, varenicline is not superior to the patch. Thus, appropriate determination of variants influencing *CYP2A6* activity is an important element in implementing precision medicine for smoking cessation. Although the genetic variants in nAChRs and the dopaminergic systems also have been associated with smoking cessation, the results from different studies have been less consistent. Further investigation is needed to improve personalized treatment. The development of treatment approaches that consider multiple genetic and environmental factors may provide important new options for the prevention and treatment of and population screening for ND. Finally, pharmacogenetic research may identify the individuals most susceptible to ND and those who may benefit maximally from certain medications.

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Chapter 20
Background, Biology and Health Concerns of Electronic Cigarettes

Abstract Electronic cigarettes (ECIGs) are growing in popularity while generating worries on the part of lawmakers, healthcare providers, and smokers. Although ECIGs supposedly are a safe alternative to tobacco smoking, they might warrant review by regulators as potentially harmful new products. Furthermore, it is possible that the use (a practice called "vaping") of ECIGs by young smokers or nonsmokers will create nicotine dependence (ND) in persons who previously were free of it. Thus, the value and risks of ECIGs are a topic of vigorous debate, particularly with the paucity of data that can tell us definitively the virtues and deficiencies of ECIGs.

Keywords Electronic cigarette · ECIGs · Electronic nicotine delivery systems · Vaping · Animal models · Harm reduction · Nicotine concentration · Nicotine dependence · Secondhand smoke · Harmful effect

1 Introduction and Background

ECIGs are unique among the smoke-free tobacco products. Their sales are increasing, often (30–50%) via the Internet (Rom et al. 2015). Based on an expected compound annual growth rate of 16.6%, the global ECIG market will reach $28 billion by 2022. The cost of traditional tobacco cigarettes in the United States was approximately $7.26 per pack in 2015, while an equivalent number of ECIGs cost only about $1.50, adding to their appeal. There are estimated 2.75 million ECIG users today, and roughly 12% of the high school-age population has tried them. Because of increasing sales of ECIGs, the US Food and Drug Administration (FDA) recently published a rule that gives it regulatory authority over all electronic nicotine delivery systems (ENDS) as well as tobacco products. Thus, the FDA now oversees the manufacture, importation, packaging, labeling, advertising, promotion, sale, and distribution of ENDS.

Some countries, such as Australia, Canada, Singapore, and Brazil, have banned ECIGs because of the lack of safety and efficacy data (Henningfield and Zaatari 2010). The first generation of ECIGs or ENDS was introduced to the market in the European Union in 2006 and in the United States in 2007. In the United States,

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ECIGs now are marketed and sold as a smoke-free tobacco/nicotine alternative to be used primarily as an aid to smoking cessation and are generally described as tobacco "harm reduction" products. However, their most popular use is as a means of obtaining nicotine.

ECIGs differ from conventional tobacco cigarettes in that they vaporize a heated fluid instead of burning tobacco. This liquid is a combination of nicotine, propylene glycol, glycol, water, and flavors. This mixture is heated by an electric device to generate a vapor that is inhaled (Fig. 20.1). Since their introduction, many changes have been made in their design and performance, including new ways to create individualized liquids, regulate the temperature, and deliver more nicotine. The versions of these products now on the market are fourth generation.

All models share several characteristics (Fig. 20.2). A cartridge transports a humectant carrier that delivers nicotine and includes propylene glycol, which often is in solution with glycerin and some flavoring. Both glycerol and propylene glycol are small chemicals that are liquid at room temperature. They are widely used as food additives and in pharmacological applications (Grana et al. 2014), suggesting they are safe in the body. The device also has an inhalation tube into which the loaded cartridge is inserted. Finally, a battery-powered heating element vaporizes the humectant to create a mist. Users of most devices can refill the cartridge as needed with "juice," the solution that contains the nicotine.

Several ECIG liquids are on the market. The quality of their production often is inadequately documented, and despite the low toxicity of glycerol and polypropylene glycol as food additives, no comprehensive data are available on the effects of their chronic inhalation. As documented in previous chapters of this book, the harm-

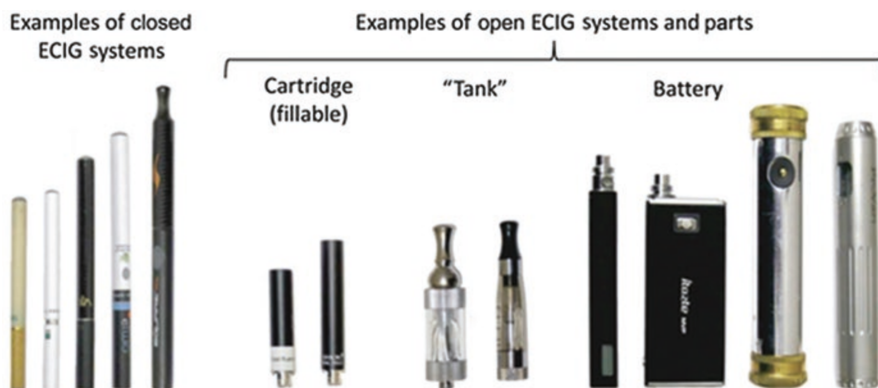

Fig. 20.1 Examples of open and closed electronic cigarette systems. Most ECIGs consist of a battery, an electrical heater, and a liquid that is aerosolized for users to inhale. There are many types. The first-generation or "cigalikes" look like cigarettes, as their popular name implies. More recent products are much larger. Some of these devices have a cartridge or tank to hold the nicotine-containing liquid. These generally have a battery separate from the cartridge or tank. Cigalikes tend to be "closed": they are not refillable. In contrast, many cartridge- or tank-based systems are "open," in that they are refillable. Many of these products allow selection and replacement of some components (Adapted from Breland et al. 2017)

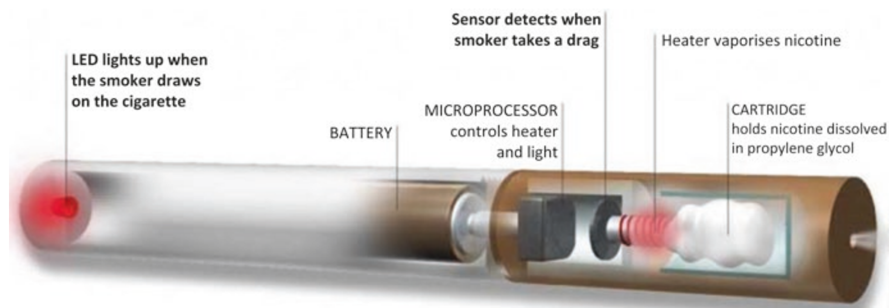

Fig. 20.2 Working of ECIGs. The battery ordinarily is the longer part of the ECIG and generally relies on lithium. The screw-on cartridge has two parts, the "atomizer" and the e-liquid container. The atomizer is a minuscule heater that vaporizes the nicotine-containing liquid into a smokelike vapor. The concentrated heat from the battery supplies the necessary energy. Most often, the battery is turned on when the user sucks on the mouthpiece as one would on a cigarette (Adapted from http://ecigarettereviewed.com/about-e-cigs)

ful properties and effects of nicotine have been studied extensively, proving its highly addictive properties and support of cancer initiation and growth. It is believed that ECIGs will be free of these risks. However, a particular concern is that the flavors included in ECIGs are not regulated in any way. Thus, although they are widely accepted as food additives, the effects of inhaling them are a mystery. Furthermore, heating of ECIGs to a high temperature can cause production of the highly toxic chemical formaldehyde (Jensen et al. 2015).

Adults report various motivations for ECIG use, including to help them quit cigarettes and to allow them to use nicotine in areas where smoking is prohibited. These are common themes in ECIG marketing and promotion. Claims of their efficacy as a smoking cessation aid have appeared in ECIG advertisements in the United States, the United Kingdom, and China. However, such claims are not yet accepted as valid by regulatory authorities. ECIGs also are marketed as a way to circumvent smoke-free policies and might be used by ND individuals who have less actual desire to quit smoking. Thus, using them as a safer alternative to cigarettes, as touted by the product advertising, is not supported by valid scientific data. Of special concern, there are no regulations governing ECIG emissions or their effects on biological systems. This is particularly worrisome in view of the questions about their safety for long-term use.

2 Issues and Concerns

2.1 Safety

As with conventional cigarettes, the safety of those in the vicinity of ECIG users must be defined. There are no standards for the identity of the ingredients, their quality, manufacturing techniques, or labeling (Callahan-Lyon 2014) even though there is significant variation among the approximately 460 brands of ECIGs on the US market. Studies that have assessed the physiological effects have found that ECIGs may be less harmful than traditional cigarettes in view of the inhaling of lesser amounts of various chemicals. Some positive physiological changes also are seen when ECIGs are used, such as reduced coughing, bad breath, sore throats, chronic obstructive pulmonary disease, asthma, and bronchitis and greater fitness. However, several negative physiological changes, such as higher blood pressure, faster heart rate, coughing, upper respiratory tract irritation, tightening of the lungs, and difficulty in breathing, have been described.

In short-term randomized trials, the use of ECIGs demonstrated no health risks (Bullen et al. 2013). Long-term damage from nicotine is slight, so any adverse health effects reported by users probably are caused by the non-nicotine constituents of the ECIG vapor. Absent long-term studies, what is known about the biological effects of the compounds in ECIG refill solutions, cartridges, and aerosols is the best indicator of the health risks with protracted use. Toxicants in various ECIG liquids and vapors include tobacco-specific nitrosamines and alkaloids, aldehydes, volatile organic and phenolic compounds, polycyclic aromatic hydrocarbons, flavors, metals, and solvent carriers (Farsalinos and Polosa 2014). These findings highlight the lack of standards in the methods used to analyze ECIG aerosols. Thus, some of tests could be underestimating or overestimating toxicant amounts and exposures. Furthermore, the papers reveal the lack of standards in the manufacturing processes used by much of the ECIG industry.

Another dimension is the possibility of harm to the nonuser population. For example, the ECIGs could introduce smoking to children and young adults, although there is little evidence that this is happening today. There also are worries about tobacco industry involvement in ECIG creation and production so that these devices could help Big Tobacco sustain the market for their original products.

Clearly, well-defined regulations must be enacted to ensure the safety of ECIGs while preventing children using from them, such as by properly directed marketing, prohibition of product sales to minors, and establishment of a regimen making safer products cheaper than harmful ones. Various regulatory arrangements are being introduced in different jurisdictions; it will be important to watch the results everywhere to see which versions are the most effective.

2.2 Nicotine Concentration

The concentration of nicotine in the "juice" in the cartridges of ECIGs ranges from 0 to 24 mg or sometimes even higher; there is a serious disconnect between the concentration listed on the label and that present in the refill cartridges. Fortunately, despite the high concentration of nicotine in the cartridges, the addiction potential is low because nicotine enters the central nervous system slowly when inhaled from an ECIG, taking several minutes, as in nicotine replacement products. In addition, the serum nicotine concentration is lower in persons who use ECIGs than in those who smoke traditional cigarettes.

3 Health Effects of ECIGs

Human exposure to some potentially harmful chemicals is significantly lower with ECIGs than with cigarettes. Laboratory analysis has found only modest increases in nicotine biomarkers after "vaping." Thus, vaping has no or only minimal impact on other physiologic measures (i.e., exhaled carbon monoxide, complete blood count, body weight), with health improvements seen in smokers switching to ECIGs, such as reduced blood pressure, improved lung function, and fewer disease symptoms. Studies measuring the cognitive effects of vaping indicate some positive impacts, including improved memory and mood, consistent with a meta-analysis of the acute positive benefits of nicotine.

3.1 Secondhand Exposure

Some studies of the health effects in non-smokers/vapers of exposure to secondhand ECIG vapor found no difference in cotinine concentrations after vapor and smoke exposures, whereas others found that although the nicotine concentration in oral fluid from persons exposed to vapor was much lower than that in those exposed to smoke, the drug was still present. Secondhand vapor studies thus indicate that non-users may be exposed to nicotine by users, although the extent of exposure to nicotine and other compounds appears to be low. Whether these concentrations are high enough to warrant biological concern is unclear, and better studies are needed.

3.2 Can ECIGs Induce ND?

ECIGs can induce ND only if they deliver an adequate dose of nicotine quickly to the brain. Cigarette smoking is still the best way to do this (Benowitz 2010). The speed and extent of nicotine delivery from ECIGs depend on the specific devices and liquids, such as battery size, device type, propylene glycol/vegetable glycerol ratio, and nicotine concentration, as well as individual user differences. Most clinical laboratory reports of experienced vapers indicated that ten puffs of nicotine-containing ECIGs can reliably increase the plasma nicotine concentration within 5–10 min, but the concentrations are significantly lower and reached a peak more slowly than is seen after ten puffs from a cigarette (Spindle et al. 2015). With second-generation devices, a plasma nicotine concentration similar to that caused by cigarettes can be achieved, depending on the user's puff topography or the liquid nicotine concentration (Lopez et al. 2016). Whether such concentrations are achieved routinely by most vapers is unclear.

So far, there is only one proposed measure of ECIG dependence (Foulds et al. 2015). Various methods of assessing ECIG dependence have been tried, and comparisons between products have been made, adapting scales for cigarette dependence or ND or measuring perceived dependence. A third of former smokers who use ECIGs daily believe they are at least as dependent on these devices as they were on cigarettes (Etter and Eissenberg 2015). However, the current ECIG products appear to be significantly less likely to induce ND than are cigarettes, although ECIGs can induce some degree of satisfaction and dependence, especially second-generation ECIGs.

3.3 Value for Smoking Cessation

A key question regarding ECIGs is their ability to facilitate smoking abstinence or meaningful smoking reduction. In some studies, ECIGs helped some adult smokers to quit or to reduce their cigarette consumption (Bullen et al. 2013; Tseng et al. 2016). The success rate was similar to or higher in the ECIG group than in subjects using nicotine replacement therapy. However, vaping may be associated simply with a change in cigarette use or demonstrate a negative correlation with cessation. Nevertheless, in studies conducted with more precise measures of ECIG use (i.e., duration, type of device, use with the aim of achieving smoking cessation), regular, more intensive vaping improved the smoking cessation rate (Brose et al. 2015). Although there are concerns about the adequacy of the experimental designs of several studies, many longitudinal studies without comparison groups, as well as cross-sectional studies, suggest that ECIGs can help smokers quit or reduce their smoking (Glasser et al. 2017). The studies describing negative correlations between ECIG use and smoking cessation have serious methodological deficiencies, in particular, selection bias (e.g., smokers who quit by using ECIGs were excluded from

the sample), inadequate measures of exposure, and confounding elements (e.g., smokers who have failed repeatedly to achieve smoking cessation are more likely to attempt quitting with ECIGs). Better-quality studies with appropriate measures and control groups must be conducted to determine whether ECIGs indeed help in smoking cessation or reduction.

4 Biological and Mechanistic Studies of ECIGs in Cell Culture and In Vivo Models

4.1 Effects on Cultured Cells

To gain insight into the biological and toxicological effects of ECIGs, various approaches have been used to investigate the effects of the liquids, as well as the vapor generated by ECIGs and inhaled by users. These studies have employed a range of target cells such as fibroblasts and endothelial, vascular smooth muscle, and embryonic stem cells (Hiemstra and Bals 2016). Differences among studies in the use of tumor cell lines, immortalized cell lines, and primary cells are of concern. Studies of airway epithelial cells that are well differentiated and incorporate various cell types, namely, basal, mucus-producing goblet, ciliated, and club cells, pose a particular problem. Primary airway epithelial cells show differentiation when cultured at an air–liquid interface, whereas most immortalized and tumor cell lines do not.

Adverse effects of ECIG vapor and liquid, such as reduced viability and greater production of inflammatory mediators and oxidative stress that would reduce antimicrobial defenses and stimulate pro-carcinogenic events, were found on airway epithelial cells and tumor and other epithelial cell lines. Interestingly, in the studies showing adverse effects, the specific damage was not mediated by nicotine alone, and some effects were largely independent of the nicotine concentration (Scheffler et al. 2015; Yu et al. 2016). This is consistent with the results of a study on the effect of ECIG liquid on human gingival fibroblasts (Sancilio et al. 2016).

On the other hand, studies on epithelial cells and a variety of other cell types demonstrate that ECIG vapor and liquid may be less toxic than cigarette smoke yet cause marked adverse effects, as judged by a variety of measures. However, the results from various studies cannot easily be compared because of differences in the types of cells studied, the exposure systems, and the ECIG brand. In addition, the lack of uniformity in generating EC aerosols hampers interpretation of the findings. Thus, further studies are needed to harmonize approaches to the investigation of the potentially harmful effects of ECIGs on cultured cells.

4.2 *Effects in Animal Models*

Animal studies are a popular tool to study exposure to cigarette smoke and chronic obstructive pulmonary disease (COPD) and cancer. Despite the utility of these models in explaining disease mechanisms, it is not clear whether the results can be translated into clinical practice. There are problems comparing results obtained in different species or with different exposure systems. Nevertheless, animal models remain valuable as a means of learning about the potential long-term outcomes of exposure to ECIG vapor. Studies to date show that exposure to vapor may produce some physiological effects such as reduced weight, increased oxidative stress, and neurobiological changes (Glasser et al. 2017).

5 Concluding Remarks

ECIGs are having an impact on public health. Additional regulatory and quality control measures need to be undertaken regarding their ingredients, quality, labeling, and manufacture. Furthermore, long-term, rigorous studies are needed to gain additional insight regarding their safety and effects on humans. Until more information is available, no conclusion can be drawn about the potential dangers or utility of ECIGs.

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Chapter 21
Grand Challenges and Opportunities for Psychiatry, Including Nicotine Addiction Research

Abstract Psychiatric genetics is a new and promising field that may reveal what genetic variants are responsible for the development of a psychiatric disorder and enable customization of drug treatment for patients with various disorders, including addiction. As discussed in previous chapters, significant progress has been made in almost every area of nicotine addiction research. However, many challenges remain. This chapter provides a list of these challenges and ways for us to attack them.

Keywords Challenge · Opportunity · Nicotine addiction · Psychiatric genetics · GWAS · Functional variants · Smoking cessation · Smoking treatment · High-throughput techniques · CpG island · DNA methylation · Smoking treatment · CRISPR/Cas9

1 Introduction

Molecular psychiatry is a still-nascent field, aspiring to imitate the great development of revolutionary tools and techniques achieved elsewhere. The rapid development of new methods such as high-throughput DNA sequencing and bioinformatics has greatly advanced our understanding of the pathology and etiology of most, if not all, psychiatric disorders, far beyond what we expected to know a few years ago.

Although these advances have brought us closer to understanding the development of many psychiatric disorders, including nicotine addiction, the majority of the questions about their origins remain. Many of these questions are not only of great scientific importance but also of broad interest to the rest of society. As reported by the World Health Organization (WHO 2008), disorders of the nervous system affect hundreds of millions of people worldwide. For example, depression is present in 154 million people, 25 million people suffer from schizophrenia, 91 million people are affected by alcohol use disorders, 15 million people suffer from drug use disorders, epilepsy impacts 50 million, and 24 million people suffer from Alzheimer's disease or other dementias. The collective burden of these disorders has a significant impact on the world's economic output.

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2 Missions

The primary missions of research in molecular psychiatry are, first, to understand the mechanisms underlying the development of psychiatric disorders at the molecular level and then to find ways to treat and even prevent them more effectively. Such broad missions imply that molecular psychiatry is not a single science but rather a multidisciplinary enterprise including diverse fields such as molecular biology and genetics, psychology and psychiatry, neurology, pharmacology, chemistry, biostatistics and bioinformatics, and engineering and computer sciences. Our past successes – in combination with the revolutionary new tools and technologies from molecular biology and genetics, information science and technology, mathematics, bioinformatics, and neuroimaging – have positioned molecular psychiatry on the cusp of great transformational progress in our understanding of the brain and how its actions result in mental activities and various disorders of the nervous system.

For decades, philosophers and scientists have argued about the influences of nurture (or the care an organism receives in its early life) vs. nature (or biological inheritance). As our understanding of the brain has advanced, it has become clear that what really matters is the interplay between nurture and nature. To the best of our knowledge, almost all common psychiatric disorders, including nicotine addiction, are complex conditions influenced by both genetic and environmental factors as well as their interactions. There is no doubt that the fields of psychiatry and studies of other nervous system disorders have benefited tremendously from better understanding of the role of genetics. However, genes are not destiny. Not only does genetics impact the development of these complex psychiatric disorders, the environment does as well. With a better understanding of brain function, i.e., the brain's ability to shape, form, eliminate, and strengthen different neuronal networks and circuits, we can begin to understand how brain structure and function change throughout our lives. What we do in brain research, more specifically in molecular psychiatry, is to determine which genes and variants are involved in a particular condition and how they are expressed during different developmental stages or interact with environmental factors to shape each person's life. To attack these daunting but important tasks, there exist many challenges, which can be summarized briefly as follows.

3 Challenges

The first challenge is to determine which genes and, specifically, which variants contribute to the development of a psychiatric disorder. Although this effort with either candidate gene-based or a genome-wide association (GWA) analysis has continued for years and has identified numerous genes and variants associated with each disorder, only a few of them have been replicated in independent samples, perhaps because of small samples (especially for the earlier studies) and

heterogeneity in defining each phenotype and outcome measure. To address those concerns, we must increase our sample size through pooled or meta-analysis and reduce heterogeneity among the samples by using intermediate phenotypes such as endophenotypes, heritable biochemical or neurophysiological markers (i.e., determined by genes), and objective measures (i.e., less influenced by behavioral factors or biased by the investigator). On the other hand, we should realize that sample size can change only the final *P* value for our results from the association test and has no impact on the effect size of each genetic variant under investigation. In other words, we should not be fooled by final *P* values and must have a proper balance between sample size (power) and the cost of subject recruitment, genotyping, and statistical analysis. Although we all agree that GWA has had some success in finding genetic variants responsible for a psychiatric disease, with the identification of variants in the nicotinic receptor subunit gene cluster on chromosome 15 that are important for smoking dependence and lung cancer being one of the most successful examples (see Chaps. 5 and 10), there are various limitations of this powerful approach. Because of the concern about false-positive findings that may result from a high-throughput approach, a stringent threshold for genome-wide significance must be adopted, in which only a few SNPs can survive correction for multiple testing, a number that appears to be far less than we would expect from quantitative genetics theory for complex traits that are determined by many factors, each with a small effect. As markers identified by GWA studies can explain only a small proportion (less than 5% in most cases) of the phenotypic variance observed (Visscher and Montgomery 2009), the hypothesis that complex diseases are attributable to a relatively few common variants has been questioned (Manolio et al. 2009).

The second challenge is to identify the functional variant(s) that causes the observed association with a disease and the molecular mechanism by which it exerts its effects. So far, most variants identified through either candidate gene-based or GWA studies are not causative. The reason for their association with the phenotype of interest is linkage disequilibrium with functional variants. This might also be one of the primary reasons for the many reported associations that cannot be replicated by other researchers with different samples. Deep sequencing of previously identified genes associated with a disease thus represents a logical step for identifying those rare variants, especially those found in families recruited for genetic studies on a specific psychiatric disease. For example, four rare variants in a candidate gene for type 1 diabetes at approximately a 1% frequency were identified through resequencing that in total contributed more to variation in the population than a single common variant in the same gene detected by a previous GWA study (Nejentsev et al. 2009). Although samples may be limited, all genetic variations (including rare SNPs, copy number variants, insertions, and deletions) identified through the 1000 Genomes Project (www.1000genomes.org) can be a useful resource. In addition to identifying functional and rare genetic variants, it is necessary to determine how they contribute to the development of psychiatric disorders, i.e., their mechanisms of action. This can be accomplished by employing conventional molecular techniques such as allele-specific expression, reporter assays, clustering regulatory interspaced short palindromic repeats (CRISPR)/Cas9 genome editing, and imaging

analysis, to name a few. Although it is easy to understand how a non-synonymous variant impacts the function(s) of a protein, it is equally important to concentrate on variants located in the regulatory regions of both the 5′ and 3′ ends of a gene, as they are more common and affect the regulation of expression of the gene by changing binding affinity of transcriptional factors or microRNAs. These are a relatively new class of small noncoding RNAs implicated in the regulation of gene expression through interaction with the 3′ end of a target RNA (see Chap. 14). For example, one recent study revealed that differential allelic expression of a functional SNP, rs686, in the 3′ untranslated region (UTR) of *DRD1* is mediated by miR-504 (Huang and Li 2009).

The third challenge is to understand how epigenetic mechanisms, which regulate gene activity without altering the genetic code, contribute the pathogenesis of diseases. Several processes, such as CpG methylation and histone modification, occur throughout a lifetime and are significant in many psychiatric disorders such as depression, drug addiction, and schizophrenia. Changes in histone modifications and DNA methylation have been found both globally and in the promoters of genes implicated in these diseases (see Chap. 17). Although genome-wide epigenetic approaches have yielded significant findings in developmental and cancer biology, such studies in psychiatric research are largely lacking.

The fourth challenge is how to utilize the knowledge and information gained from genetic/genomic studies in practice. There are at least two aspects of this challenge. The first is related to drug discovery based on validated molecular targets. Given the complexities and high degree of difference in genetic variants, preclinical models to assess drug efficacy, and human trials with appropriate statistical power, we need to develop a system-wide approach to facilitate the translation of basic discoveries into validated drug targets (Conn and Roth 2008). The second aspect is related to genetic testing and personalized (precision) medicine. Although it may be difficult now to implement genetic testing in prevention programs for psychiatric disorders because of ethical concerns and the availability of only limited causative variants in genes implicated in a disease, the prospect of using genetic information to tailor medical treatment for psychiatric disorders is exciting (see Chap. 19).

The final challenge is how to handle the massive datasets and other information collected by those approaches. With the advance of technology, enormous quantities of data can now be generated quickly from GWA studies, RNA expression studies based on microarray and RNA sequencing, genome-wide studies of changes in methylation and histones, and deep sequencing for both SNPs and copy number variants of whole genome or candidate genes implicated in psychiatric illnesses. Unfortunately, only a few research laboratories are staffed and equipped with both hardware and software for such challenges. We need collaborations among molecular biologists, biostatisticians, and computer scientists to find effective means/tools, not only to manage the data but also to analyze and interpret them.

4 Concluding Remarks

In sum, we have made significant progress toward our goals; however, we still have a long way to go. Yes, these are challenges but also opportunities. Much work needs to be done, not only to determine which genetic variants are involved and how they are engaged through what mechanisms but also how to translate these basic science advances into new therapeutic options for the prevention and treatment of psychiatric disorders.

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