A. Candidate Background.

The overall goal of my K01 Mentored Research Scientist Development Award (MRSDA) Application is to become an expert in applying translational methods of genetics and neuroimaging to study the etiology of substance use and its related behavioral disorders. My proposed research plan focuses on the relationship between maternal prenatal smoking and offspring behavioral psychopathology, which will provide an excellent model through which to obtain the skills I seek, while at the same time addressing mechanisms underlying a serious public health problem. The conjunction of the research and training plans will provide me the skills to expand my investigation to other exposures, genes, and behavioral outcomes relevant to addictive disorders.

I bring to this application strong backgrounds in functional brain imaging research (through my doctoral training at Cornell University) and in the epidemiology of psychiatric disorders (through my post-doctoral training in the Department of Psychiatry at Columbia University), which are documented in a number of publications: I have authored one book chapter[1] and 14 original manuscripts[2-15], including five as first-author[10-14], one of which[10] was selected by *Molecular Psychiatry* [the journal with the second highest impact factor [12.8] of all Mental Health Journals] for Immediate communication. I have presented my research at a number of symposia and Grand Rounds, and have received Travel Awards from the *American College of Neuropsychopharmacology (2007)* and the *Organization of Human Brain Mapping* (2001 & '02), as well as both a NIDA funded Travel Award (2008) and an Early Career Track Investigator Award (2009) from the *World Congress of Psychiatric Genetics*. Finally, I have received independent funding in the form of a 2007 NARSAD Young Investigator Award, and, in July 2009, a one-year Research Associate Award from the New York State Psychiatric Institute to pursue my investigation of smoking and substance use disorders.

Even though I bring to this application strengths in psychiatric research, I need to (1) develop further expertise in the phenomenology, assessments, and study designs pertaining to substance use and its related syndromes. This will thus form a backbone of my 5-year training plan. In addition, I will seek (2) training in genetics, including methods of genetic analysis and the integration of imaging and genetic data, as well as (3) further methodological expertise in the use of longitudinal epidemiological and healthcare data to address trajectories of smoking and substance use. Finally, I will continue to receive training in the ethical conduct of research. The following sections detail my career objectives, how my cumulative academic experiences have led to and shaped this application, and how the proposed training plan will build upon these experiences to ferment an independent career. Should this award be funded, I will commit to spend no less than 75% of my time and effort in any given year on this project.

B. Career Goals and Objectives

B1.Doctoral Training in Brain Research. My Ph.D. training (at Cornell University Weill Medical College, under the mentorship of Joy Hirsch, PhD) focused on the use of functional Magnetic Resonance Imaging (fMRI) to study mechanisms of sensory integration within the brain. My undergraduate and graduate coursework in the neurosciences had piqued my interest in brain mechanisms, and I was intrigued by how information from the outside world could be so elegantly yet parsimoniously encoded within the brain. I began my rotation on a project that examined how hot and cold thermal sensations are represented in the cortex. We showed that mapping of temperatures within the somatosensory cortex was distinct from the mapping of body parts, suggesting a distributed system for thermal versus tactile processing[2]. For my doctoral dissertation thesis, I chose to examine how different modalities of sensory information are recruited by the frontal cortex for decision-making functions. By designing a paradigm that allowed dissociation of decision-specific from sensory-specific processes, I identified regions within the medial frontal cortex (notably, the supplementary motor area and anterior cingulate cortex) in which activation patterns varied based on the type of information contained in the decision task. For example, I found that behaviors requiring processing of spatially oriented information activated predominantly right hemispheric supplementary motor areas, whereas those requiring processing of temporal or object related information recruited homologous left hemispheric areas***,*** *regardless* of whether the input came from visual, auditory, or somatosensory cortex. These findings, published in the *Journal of Cognitive Neuroscience*[11], were noteworthy in that we showed how information within the brain can be broken up and repackaged based on decision-relevant cues. I followed this with a second study, published in *Perceptual and Motor Skills*[13], showing that tasks requiring fine-tuned coordination recruited cortical circuits differently based on the sensory modality used to guide the task.

The above studies trained me in the principles of MRI and functional MRI (fMRI), as well as with the latest analysis tools. But they also, more broadly, instilled within me an appreciation for *systems-level* approaches—that is, examination of the brain as an integrated whole rather than a series of functionally independent units. This appreciation has served me well in my transition to psychiatric applications, where I very quickly learned the complexities of behavioral phenotypes, and of their likely origins from complex regulatory circuits rather than discrete regional abnormalities.

B2. Post-Doctoral Research: From Normal Functioning to Behavioral Disorders. Toward the tail end of my doctoral dissertation in 2004, I had the opportunity to meet Myrna Weissman, PhD at Columbia University. Having focused on mechanisms of cognitive functions in healthy volunteers thus far, I was interested in applying my training to study behavioral psychopathology. Dr Weissman, meanwhile, was looking to integrate imaging modalities within her long-term epidemiological cohort studies. I remember remarking to myself after our very first conversation what an ideal opportunity this could be, and was fortunate to be invited in January 2005 to join her Division (Clinical and Genetic Epidemiology) to work on a number of on-going projects of anxiety and depression. I received the title of Post-Doctoral Research Scientist and have since been successively promoted to Associate Research Scientist (2007) and Assistant Professor of Clinical Neurobiology (in Psychiatry) (2009). Because I was originally funded by specific research protocols rather than an NIH training grant (I was not yet a US Permanent Resident at the time, and thus not eligible for the latter), Dr. Weissman's role was originally that of an employer rather than a mentor, and she could not provide protected time for systematic training or coursework. Yet, I was provided an opportunity for learning, and was encouraged to develop my own research projects and write my own grants. I realized how much more I could draw from her expertise in a formal mentorship capacity, and my decision to now have her play the leading mentoring role stems not only from her expansive scientific knowledge and resources, but also from the valuable and unflinchingly generous guidance she has provided me thus far.

At the time I joined her division, a large NIMH Program Project grant on the genetics of anxiety disorders was underway. Clinical data collection was in its last year, and analyses were about to begin. Understanding that this would provide a great hands-on learning experience and serve as a springboard to launch my own studies, Dr. Weissman also offered me the opportunity to coordinate this study. I thus quickly became proficient in methods of screening, recruiting, interviewing, pedigree collection, data management and tracking, and IRB/use of human research subjects. That behavioral disorders often seemed to lack clear boundaries only made them more interesting to me, and I supplemented my hands-on training with courses in Epidemiology and Biostatistics, and attendance at the ACNP and APPA meetings. I also became well-versed with a variety of diagnostic instruments, as the project assessed not only formal DSM based diagnostic categories, but also symptom, family history, behavioral, and early developmental measures. This program

project was thus fundamental in accelerating my learning curve in the methods of epidemiologic research, and in orienting my perspective toward pathological applications. It was also highly productive, yielding two first-author peer-reviewed papers in top-ranking journals[10, 12]. In the first, published in *Biological Psychiatry*[12], I presented family data on a syndrome of related clinical symptoms related to autonomic dysfunction that previous reports from our group had suggested may arise from a pleiotropic mechanism linked to chromosome 13[16-19]. The second, published in *Molecular Psychiatry*[10], *c*ompared a control population that we had recruited with a larger NIH repository control sample to determine comparability and to address the larger debate in the field over the relative importance of sample size versus phenotypic homogeniety. Our findings suggested the importance of the latter, by showing that NIH sample was only comparable to our interviewed controls when restricted to subjects free of any self-reported psychiatric or substance use symptoms, *regardless* of the disorder being addressed. These findings shed an important light, as the NIMH controls were collected in a repository for use by genetic investigators, but have never been directly interviewed. The manuscript was selected by *Molecular Psychiatry* for Immediate communication [defined by the journal as "*definitive studies of high merit, exceptional significance and novelty, which warrant rapid dissemination"*], and was further noted by the Editor as being one of "only a handful of manuscripts" he had accepted in his 12 years at the journal without requiring any revisions whatsoever.

Finally, even though the program project was to study the genetics of anxiety disorders, I was encouraged to develop an imaging supplement on a sub-sample, and received a NARSAD Young Investigator Grant to examine brain circuits related to consciously and unconsciously perceived fear among subjects with panic and social anxiety disorders. Even though subjects with anxiety disorders are difficult to recruit for imaging studies, I was able to recruit and scan 53 subjects (more than the originally planned sample of 45). Recruitment is now closing and I have begun analyzing the functional as well as structural data, with the goal of having a submitted manuscript by December 2009. Even though the outcomes of this study were on anxiety rather than externalizing behaviors, the experience in conducting a study from beginning to end, as well as analyzing the data (the imaging modalities and analysis software package are the same as those proposed in this application) will serve me well during the course of this MRSDA period.

B3. Focus on Substance Use and Smoking. Although the above program project served as a valuable training vehicle, it was Dr. Weissman's three-generation longitudinal study of families at high or low risk for depression (hereon, "3-Generation study")[20, 21] that stimulated the path that has culminated in this K01 application. This is a unique sample, comprising three successive generations, the first two of which have been followed prospectively for up to 25 years on a wide battery of diagnostic, behavioral, and environmental measures (more fully described in the Methods, Section D.1). Over recent years, imaging, electroencephalographic (EEG), and genetic biomarkers have been successively introduced. Though the focus of the study has not been on substance use, diagnostic information on drug and alcohol related disorders has been assessed as part of the clinical interviews at each wave. Unfortunately there are no data on smoking, except within the context of smoking by mothers during pregnancy, which were collected as part of parental medical history. Dr. Weissman's group had reported ten years ago that offspring exposed to prenatal nicotine were at increased risk for drug and alcohol use problems. At that time, many of the offspring had not passed the age of risk for these disorders, and there were no genetic or imaging markers to test underlying mechanisms. When Dr. Weissman received a supplement from NIDA to follow up on the earlier findings, I was very interested in leading this focus as I could not only follow the offspring through the age of risk for these disorders, but the availability of MRI data could allow me to me to integrate neuroscience with epidemiology to test how development of brain circuitry could predispose to these problems. When I first examined the longitudinal data that covered until the present wave, I found even stronger associations between maternal smoking during pregnancy and a range of long-term offspring problems including conduct disorder and drug and alcohol use disorder. These associations were independent of numerous potential confounding variables (discussed in Preliminary Studies; Section C1), and appeared specific to prenatal exposure. That the effects of prenatal insults persist much later than the window of exposure was particularly intriguing *neurodevelopmentally,* as it suggests that the toxic exposure is somehow altering brain development in ways that have lasting or delayed behavioral effects. And the known ability of tobacco ingredients to interfere with neurotransmission (discussed in section B of the research plan) provided an opportunity and framework to apply my imaging expertise to develop a program to test behavioral circuits in the brain and whether disruptions to these circuits might mediate the long-term behavioral problems resulting from *in utero* nicotine, drug, or alcohol exposures.

Because the 3-generation study had not been designed to study substance use, there were a number of limitations that I would need to address in order to undertake my study optimally. For example, there are currently no outcomes on smoking (except within the context of pregnancy) or on any adult behavioral measures related to substance use or smoking; a number of offspring had not passed through the age of risk for substance use disorders at their most recent assessment; and because brain images were always collected at the most recent clinical assessment, it is possible that detected changes in brain function or even structure may reflect compensatory rather than causal mechanisms (this is particularly true for drug and alcohol related disorders, where chronic use can result in cerebral atrophy[22]). I have therefore proposed a new wave of assessments (detailed in Methods, D2) that will follow offspring prospectively on drug, alcohol, and nicotine use and their disorders, as well as behavioral measures of impulsivity and aggression. This schedule will allow me to follow subjects who had not previously passed through the age of risk for substance use, to study a broader range of externalizing traits and behaviors, and generate sub-groups of subjects who develop externalizing disorders *after* the scan (thereby providing a cleaner meditational framework with which to separate causal from compensatory brain changes.)

I believe that this "hybrid" approach is optimal to my research and training mission. On the one hand, I have already had substantial experience in the mechanics of running a study— from IRB development, to recruitment, screening, scanning, data analysis, and reporting of findings. I also have several years of functional imaging experience on both psychiatric and non-psychiatric populations. I do not believe that it would be a productive utilization of the training period afforded by this award to recruit or image a *de novo* study sample, and were I to do so, any such sample would necessarily be limited in scope. The proposed study will allow me to focus on, and develop expertise in, assessments of substance use and its related behavioral disorders, and to assess subjects on these outcomes of interest, while at the same time availing of the richness, longitudinal design, and brain scans, of the parent study. Data generated in the course of this award period will lead to further R01 applications targeting specific behavioral circuits and their role in psychopathology.

B4. Focus on Genetics Functional neuroimaging, the modality through which I entered the field of mental health research, is unquestionably a powerful tool in that it allows identification of brain abnormalities that might underlie behavioral problems. But it does not the answer "upstream" questions: i.e., what predisposes to these brain abnormalities in the first place? Because brain structure and functional circuitry are themselves in part genetically determined, combining measures of genetic variation with imaging approaches— exemplified by the relatively new but provocative field of imaging genetics[23, 24] — may allow us to more comprehensively probe behavioral circuitry. To become an expert in imaging genetics, I realized I would require additional training in genetic analyses and in the integration of imaging and genetic modalities. Acquiring this training would not only serve this specific project, but also allow me to expand the focus to other genetic variants implicated in other disorders and behavioral circuits. I began by familiarizing myself with the genetic literature relevant to nicotine and substance use, and explored the genetic data that were beginning to be acquired through the 3-generation study. Though genotypes were only available on a sub-sample, I first analyzed serotonin transporter and receptor variants under the supervision of Dr. Hamilton. Though we did not detect any associations with the well known serotonin transporter polymorphism[25], the serotonin 1B receptor (HTR1B) gene was highly associated with a number of behavioral and substance use phenotypes, and further appeared to moderate the association between prenatal smoke exposure and offspring CD (detailed in preliminary data section C2). I presented the initial findings at the annual meeting of the *American College of Neuropsychopharmacology*, and received a NIDA funded award from *International Society for Psychiatric Genetics* to present these findings at the 2008 annual meeting. As DNA collection is nearing completion, I will submit the findings for publication once genotyping is complete.

My continued dedication to this line of research is evidenced by a number of recent developments. First, I received two awards within the last 3 months— an Early Career Investigator Track Award from the *International Society for Psychiatric Genetics* and a Research Associate Award from the *New York State Psychiatric Insti*tute— both intended to continue translational studies of smoking and substance use. And second, I have already, in conjunction with my co-mentor, Dr. Hamilton, begun a review of the genetic literature, and have identified additional variants within the nicotinic and dopaminergic family that we will test. Both gene families are relevant to substance use and addictive disorders. The many dopaminergic gene variants are further attractive from a neurobiological and neuroimaging perspective, as they are differentially expressed through frontal and sub-cortical regions[26], and variation in the respective genes could lead to differential neurodevelopmental (and ultimately behavioral) impairment, which I will be able to test. This

expanded focus will allow me to examine a broader range of genetic variation and its role in behavioral circuits, and to obtain additional fluency in genetic methods, including methods of multi-gene analysis (e.g., epistatic interactions).

B5. Conclusion. My long-term goals can be summarized by the title of a review manuscript I am currently writing with Drs Myrna Weissman and Alan Brown: "Translational Epidemiology". In this article, we make the case that though epidemiology has played an important role thus far by identifying modifiable risk factors that have led to important prevention and intervention strategies, the epidemiology of the future might be of greatest utility when its many design strengths are coupled with biological approaches to target etiological mechanisms and examine the roles of biology and environment. My proposal attempts to do just that: I have taken a well-known epidemiological finding (that prenatal smoking leads to offspring substance use and behavioral psychopathology) and propose to test how genetic and neurobiological variation can modify this association and thereby offspring risk. This translational approach, I believe, is well matched to the National Institute of Drug Abuse's own interests in supporting "*a spectrum of research and research training programs that addresses relationships among drug use/ abuse/ addiction, social/physical environment factors, and human development, with the emphasis on neuro-developmental, cognitive, and behavioral mechanisms that underlie these relationships*".[27]

C Career Development/ Training Activities during the Award period

C1. Rationale and Career Goals: My training goals are designed to develop skills in the following areas: (1) to develop additional expertise in the etiology, assessments, and study designs pertaining to substance use and related externalizing behavioral disorders; (2) to obtain additional fluency in genetic analyses, and in the integration of genetic with imaging data; (3) to obtain expertise in the use of longitudinal epidemiological and healthcare datasets, particularly those relevant to smoking and substance use; and (4) to obtain continued training in the ethical conduct of research. The first two goals are significantly larger in scope, and time and resources will be devoted accordingly.

C2. Rationale for Selection of mentors and advisors

Myrna M. Weissman PhD will serve as the primary mentor for this K01 proposal. She is currently Professor of Epidemiology in Psychiatry at Columbia University College of Physicians and Surgeons and the Mailman School of Public Health, and the Chief of Epidemiology at New York State Psychiatric Institute. She is a world-expert in psychiatric epidemiology, with more than 500 publications. She has been the Principal Investigator of several large clinical, genetic, and most recently imaging studies, and has mentored a number of K awardees for over thirty years, many of whom have since had highly successful independent careers of their own, including two (Bruce Rounsaville and Edward [Ned] Nunes) in the field of substance abuse. Dr. Weissman will serve as my overall mentor, guiding and supervising my research plan, and advising on epidemiology and incorporation of biological and epidemiological approaches. She will provide me access not only to the 3-Generation Study (which I will use to build my study sample) but also a number of other valuable genetic samples she and others in her Division have collected, in order to replicate and extend my findings. She will also direct me toward appropriate experts and resources as needed, and advise on publications and preparation of follow-up R01 applications that stem from this award period. Her office is in the same building as mine. In her capacity as my primary mentor, she and I will have weekly scheduled meetings to discuss the research plan. She will be responsible for ensuring completion of each of the training goals discussed below. In addition, we will have twice yearly conference calls involving all mentors to track my overall progress and discuss future strategies. Co-mentors will be asked to provide a progress report to Dr. Weissman on an annual basis, based on which formal feedback will be provided to me, and also included in the annual progress report.

Targeted instruction from the following faculty members will supplement my mentorship with Dr. Weissman.

Training in Substance Use: Drs. Hasin and Levin will serve as co-mentors, focusing on epidemiological and clinical areas respectively. Dr. Brown will advise on prenatal substance epidemiology.

Deborah S. Hasin, PhD is Professor of Clinical Epidemiology at the College of Physicians and Surgeons and the Joseph L. Mailman School of Public Health at Columbia University, and at New York State Psychiatric Institute. Her research covers several areas, including gene-environment interaction, nosology, and the nature of comorbidity between substance and psychiatric disorders. She is an internationally recognized expert who is a member of the American Psychiatric Association DSM-V Workgroup on Substance Use Disorders, Associate Editor of Drug and Alcohol Dependence, and a member of the National Advisory Council of NIAAA. She directs the Substance Dependence Research Group in the Division of Clinical Phenomenology at New York State Psychiatric Institute. Dr. Hasin has received extensive research funding from NIDA and NIAAA, and has authored over 200 publications. She has mentored a number of NIDA K awardees and her diagnostic research interview, the PRISM, is in use in numerous studies of the relationship of substance and psychiatric disorders.

Frances R. Levin, MD is the Kennedy Leavy Professor of Clinical Psychiatry at Columbia University Medical Center, the Director of the Addiction Psychiatry Fellowship, at New York Presbyterian Hospital, and Director of Clinical and Educational Activities for the Division on Substance Abuse, New York State Psychiatric Institute. Dr. Levin is an expert in the study and treatment of substance use disorders as well as the relationship between substance use and other psychiatric illnesses. She has published over 100 manuscripts, and has mentored a number of K Awardees.

Alan S. Brown, MD MPH is an Associate Professor of Clinical Psychiatry and Epidemiology at the College of Physicians and Surgeons of Columbia University, New York State Psychiatric Institute and the Joseph L. Mailman School of Public Health. He is the Director of the Unit in Birth Cohort Studies in the Division of Epidemiology at NYSPI. His primary research interests lie in the identification of prenatal and early developmental risk factors for psychopathology. He was the first to demonstrate that serologically documented

prenatal exposure to influenza, rubella, toxoplasmosis, and inflammatory markers are potential risk factors for schizophrenia, and is currently involved in several large birth cohort studies.

Training in Genetics: Dr. Hamilton will serve as a co-mentor. Dr. Hodge will serve as an Advisor for Statistical Genetics, and Dr. Hariri, at Duke University, as a Consultant for imaging genetics.

Steven P. Hamilton, MD PhD is Associate Professor of Psychiatry and the Carol Cochran Schaffner Endowed Chair in Mental Health at the University of California at San Francisco (UCSF). He is a Psychiatrist and Molecular Geneticist, and his primary research area is in the genetics of psychiatric disorders, and in using molecular genetic techniques to map genes for psychiatric disorders. Even though he is not physically located at Columbia University, he has had a long and extensive track record of publications with my primary mentor, Dr. Weissman, and with Dr. Hodge (Advisor), and is also the Three-Generation Study Cohort, on which my proposal will build.

Susan E. Hodge, DSc is Professor of Clinical Biostatistics at Columbia University and an expert in mathematical modeling of genetic data. Dr. Hodge is founding member of the International Genetic Epidemiology Society, the Associate Editor of Human Heredity, and Principal Investigator of a T32 Training Grant that focuses on training young investigators in the genetics of complex disorders. She has published extensively with both Drs. Weissman and Hamilton.

Ahmad Hariri, PhD is Professor of Psychology & Neuroscience and Investigator in the Institute for Genome Sciences & Policy at Duke University. He previously served as the director of the Developmental Imaging Genetics Program at the University of Pittsburgh. Dr. Hariri is a pioneer and one of the foremost experts in the field of imaging genetics, and has published in all the leading journals in the field, including *Science and Nature.* His research is focused on using modern molecular genetics and neuroimaging methods to identify specific biological pathways that help shape individual differences in temperament and personality, and he has ongoing imaging genetic studies focusing on the development of substance dependence in adolescent girls, as well as on risky decision making.

C3. Training Plan. The training plan for each goal is detailed below, and then summarized in **Tables 1 and 2** that follow. The training directed at specific goals will be enriched by my regular attendance at Grand Rounds, conferences, and journal clubs, as described below.

Goal 1. Training in substance use and related behavioral disorders.

Rationale for Training: I seek to obtain proficiency in the etiology, phenomenology, and assessments of substance use disorders (including drug, alcohol, and nicotine use). Given my focus on translational methods, I also believe it is vital to be fluent not only in formal substance related diagnoses, but also with a spectrum of behavioral disorders and traits (in both child- and adulthood) that are related to, and may predispose to addictive disorders. There are multiple reasons for this: (1) From an *epidemiological* perspective, behavioral disorders and traits may be detectable earlier than diagnostic outcomes and can provide important information on early risk factors for addiction. (2) *Biologically,* behavioral traits may be more proximally allied to the underlying genetic and neurobiological architecture than syndromes of abuse and dependence that are more fluctuant to external variation (e.g., family environment, access), and may thus be easier to detect, particularly in genetic and imaging studies. And (3) *statistically,* quantitative trait measures carry greater power.

A substantial focus of my training will be on assessment methods and instruments. Although I am familiar with, and have used, semi-structured interviews (e.g., the SADS) to assess DSM-based categorical disorders, I need additional exposure to finer-grained assessments of drug and alcohol use (including smoking) as well as behavioral measures. I am fortunate to have the mentorship of Dr. Hasin, who is an internationally renowned expert. She is currently a member of the American Psychiatric Association DSM-V Workgroup on Substance Use Disorders, has chaired the Measurement Group for the national NIDA Clinical Trials Network, and provides consultation on measurement issues in drugs, alcohol and psychiatric comorbidity to many other investigators. Through scheduled in-person tutorials with her, I will learn about the various instruments, their psychometric properties, and appropriate usage. We will also review the literature on substance use disorders to identify candidate genes which I will then be able to target in imaging studies. I will also take her course on the epidemiology of drug and alcohol use disorders, which will include a substantial focus on etiology.

I will also seek to obtain additional training in clinical and behavioral phenomenology of substance use disorders so that I can more acutely target brain circuitry related to addiction in subsequent study designs, as well as biological indices of drugs, alcohol, and nicotine, as these can provide additional objective phenotypes

to supplement behavioral and clinical measures. Here I am fortunate to have the mentorship of Frances Levin MD, who is an expert in clinical studies of substance use, and will provide tutelage in these areas. Additionally, though the current research proposal is based on a non-clinical sample, follow up studies will target genetic and neurobiological correlates of clinical features such as symptom severity, treatment response/resistance, tolerance, withdrawal. This is an area I am already familiar with, having published a manuscript on factors moderating remission from depression in the STAR*D Child Study[14]. I will therefore perform a 6-month bi-weekly observership at Substance Treatment and Research Service (STARS) under the guidance of Dr. Levin, who will select and guide my participation in selected on-going studies. Finally, I will participate (based on topic relevancy) in the Substance Abuse Fellows Seminar, also directed and moderated by Dr. Levin.

Through my interactions with Drs. Hasin, Levin and Dr. Weissman, I will develop a keener understanding of comorbidities between substance use, psychiatric, and general medical conditions. This is an important focus as comorbidities with substance use are common[28, 29], and failure to account for them, particularly in translational studies, can easily lead to erroneous interpretation of the etiology. All three of these mentors have studied and published prolifically on the relationship between substance use and psychiatric disorders, and Dr. Hasin's Psychiatric Research Interview for Substance and Mental Disorders (PRISM) is used internationally in studies focusing on substance use and psychiatric comorbidity.

Finally, I will devote a portion of my training to learn about prenatal substance exposures and their relation with psychiatric outcomes. I will achieve this via a combination of course-work and one-on-one meetings with Dr. Alan Brown (also see Goal 3), who is the Director of the Unit in Birth Cohort Studies at NYSPI, and an expert in examination of prenatal factors and their outcomes. He will help me to interpret my findings in the context of epidemiological literature on prenatal risk factors and to better understand how *in utero* influences such as smoking impact on prenatal development to lead to delayed onset psychiatric disturbances.

1. Formal Coursework (*All Courses at Columbia University*).

a. Epidemiology of Alcohol and Drugs (P8470). This course focuses on disorders of drug and alcohol abuse and dependence, and includes main studies in each area and discussion of etiology, pharmacology, and psychosocial and genetic factors, with discussion of treatment.

b. Perinatal Epidemiology (P8422). This course covers teratogenics, reproductive loss, birth weights, and pre- and perinatal infections.

2. Mentorship and Hands-on Practicum

a. Biweekly scheduled meetings with Dr. Levin*: clinical and behavioral attributes; study designs; selection and supervision of STARS participation.

b. Monthly scheduled meetings with Dr. Hasin*: discussion of assessment instruments, epidemiologic studies focused on etiology.

c. Monthly scheduled meetings with Dr. Brown*: discussion of prenatal exposure epidemiology (also Goal 3).

d. Observership (bi-weekly, for 6 months) in the Substance Treatment and Research Service (STARS).

**Regularly scheduled meetings through Years 1 and 2, then as needed.*

3. Seminars, Journal Club and Scientific Conferences (These encompass all goals).

(a) Seminars: I will attend weekly the New York State Psychiatric Institute / Columbia University Department of Psychiatry, Grand Rounds, which routinely host the world's experts in the field of Psychiatry, and have included a significant number of topics related to genetics as well as brain-behavior interface. I will also attend, based on topic relevancy, the Substance Abuse Fellows Training Program Weekly Seminars, led by Dr. Levin, the Department of Child Psychiatry Grand Rounds, the Department of Biostatistics Journal Club and the MRI Journal Club.

(b) Conferences: I will attend, and present annually at the Annual meeting of the American College of Neuropsychopharmacology (ACNP), as I have done for the last three consecutive years. I will also attend other conferences including the College for Prevention of Drug Dependence (COPD) or World Congress of Psychiatric Genetics (WCPG).

Goal 2: Training in Genetics, including analysis of genetic data and integration of genetic data with clinical and neuroimaging data.

Rationale for Training: Individual genetic variability plays a substantial role in behavioral regulatory circuits, which in turn predispose to behavioral problems and substance use. Genetic and imaging approaches

can together test questions that either alone may not. Although I will not be performing the laboratory genotyping work myself, it is essential that I fully understand multiple genotyping methods- including the laboratory processes entailed- and how to appropriately analyze genetic data. This training in genetic methods will be through a combination of formal didactic coursework at Columbia University Medical Center, and one-on-one mentorship Dr. Hamilton. I will additionally consult with Dr. Hodge on issues of statistical genetics, and with Dr. Hariri on the latest analysis methods for integrating genetic and imaging data, as well on the latest functional imaging paradigms to target externalizing behaviors.

I have also begun, under the mentorship of Drs. Hamilton and Hasin (who will also contribute to Goal 1), to review the genetic literature on substance use disorders and their behavioral counterparts, in order to identify novel polymorphisms of interest. These will augment my training by allowing me to subsequently explore a broader range of genetic variation, including gene-gene interactions, and obtain pilot data during this award period to direct subsequent R01 applications. I will also, in the second or third year of my proposal synthesize this review in a formal manuscript. I have a track record to support this effort, having completed a comprehensive genetic review of panic disorder for a textbook, *Psychiatric Genetics,* that will be published in 2010 by Cambridge University Press[1].

1. Formal Course Work

a. Methods in Molecular/Genetic Epidemiology (P9407): Focuses on methodological issues pertaining to genetic/non-genetic biological markers.
b. Neurobiology and Genetics of Psychiatric Disorders (P8419): Focuses on biological and genetic factors in the development of psychiatric and substance use disorders.
c. Genetic Analysis Laboratory (P8141): Hands on course using computer simulations to model genetic data. Topics include basics of linkage analysis, mode of inheritance assumptions, heterogeneity, complex models, ascertainment, and multipoint analysis.

2. Mentorship and Hands-on Practicum

a. Monthly scheduled conference calls with Dr. Hamilton to discuss progress in genotyping, and analysis of genotyping data; discussion and review of the literature and identification and selection of novel polymorphic variants of interest to substance use and externalizing disorders.
b. One 3-day visit to Dr. Hamilton's laboratory at University of California San Francisco (2nd Year), to participate in complete genotyping experiment and data analysis.
c. Quarterly conference calls with Dr Hariri to discuss analysis strategies and obtain feedback for integrating genetic and neuroimaging data.
d. One 2 day visit to Dr. Hariri's laboratory at Duke University (3rd Year) to learn about advanced methods of analysis including whole-genome and whole-brain independent component analyses.
e. As needed one-on-one meetings with Dr. Susan Hodge, discuss statistical analysis of genetic data.
f. Twice yearly group conference calls with all mentors, co-mentors, advisors, and consultants, to discuss overall progress and chart course of the upcoming months.

Goal 3: To gain experience in the use of longitudinal datasets relevant to smoking and substance use

Rationale: This goal is particularly important in the context of substance use, where, because exposures cannot typically be experimentally randomized, large population-based cohorts with prospectively collected data are ideally required in order to address several sources of bias including confounding. On the other hand, however, these large population-based samples (at least until very recently) have not carried detailed biological markers and therefore are not malleable to translational studies. As I became more involved with the 3-generation study and the literature on studies of substance use, it seemed that a complementary approach – i.e., coupling more focused samples to target specific biological substrates (such as this proposal entails) with larger population-based studies to ask broader epidemiological and clinical questions— can provide the greatest information. I have therefore asked Dr. Alan Brown, who is an expert in using large birth cohorts to study prenatal risk factors, to serve as an Advisor on this proposal. One study that we have already identified through our preliminary discussions is a large population based Finnish national birth cohort, which contains archived prenatal serum specimens from which detailed information on multiple maternal toxic prenatal exposures, including a biomarker of nicotine, can be obtained, a large database including maternal smoking during pregnancy, as well as detailed longitudinal assessment of offspring outcomes. The database covers all of Finland (and is therefore generalizable), with data on ~ 60,000 mothers who smoked during pregnancy over the last 25 years. I will meet with Dr. Brown in one-on-one supervision to expand my knowledge on the

potential role of environmental exposures such as smoking in psychiatric disorders and public health interventions, and on the design and implementation of studies aimed at addressing these questions. We will compile a literature review on prenatal nicotine exposure and developmental/neurodevelopmental outcomes, other prenatal exposures and psychiatric disorders including the work of Dr. Brown's team and other research groups, and the impact of public health interventions including smoking reduction on disease outcomes. We will also discuss potential approaches that will provide the basis for me to investigate the question of prenatal smoking and psychiatric outcomes in future work on birth cohort studies, including the Finnish birth cohort. I will also attend Dr. Brown's research meetings in his Unit on Birth Cohort Studies. Importantly, though not currently available, permissions to obtain genetic materials as well as brain scans can be applied for, and this cohort could serve not only to replicate findings from the present study, but also to test interactions of several other exposures with genetic factors. We will also identify other studies, targeting those with either existing genetic and/or imaging data, or the potential to incorporate such data in the future. Though these additional studies do not comprise my primary research plan, we will identify variables of interest and obtain preliminary frequencies as appropriate to the goal of targeting these samples in subsequent R01 applications.

1. Formal Coursework

a. Use of Large Scale National Health Care Datasets (P6781) This course discusses research methodologies pertaining to health care data sets, including federal, state, and local level resources. Covered are (1) variable identifications and definitions; (2) record layouts; (3) data set size and analysis restrictions; (4) variable strengths and weaknesses; (5) research protocol submissions required by agencies for access to confidential data; and (6) data handling methods.

b. Analysis of Repeated Measurements (P8157) This course covers features of repeated measurements studies, time-varying covariates, correlation structure, random effects and mixed models, growth curve and autoregressive models, non-parametric approaches and models for repeated binary data, and applications of generalized linear models to repeated data This course will be relevant not only in terms of the Finnish Birth Cohort and the 3-Generation Cohort, but also for future birth cohort or other longitudinal studies.

2. Tutorials and Hands-on Practicum

1. Monthly scheduled meetings with Dr. Brown (also see Goal 1); coupled with attendance at Birth Cohort Studies Meetings.
2. Participation in the monthly meetings of the Sackler Institute for Development Psychobiology at Columbia University. The Sackler group, under the stewardship of Jay Gingrich MD PhD, Alan Brown MD MPH, Myrna Weissman PhD, and a number of other investigators (including Drs. Hamilton and Hodge- Goal 2), is embarking on a study of outcomes related to prenatal SSRI exposures. By considering prenatal smoke exposure in the same sample, I will be able to draw from these distinguished investigators while at the same time developing my own expertise and sketching my own future research plans. A corollary point: the Sackler laboratories also encompass mouse and baboon research groups, and while I do not aim to develop expertise in animal models, my attendance will allow me to learn more about translational research and how clinical phenotypes can be informatively modeled across species.

D. Training in the Responsible Conduct of Research

I have already completed courses, and obtained the respective certificates, for courses in Columbia University Good Clinical Practices and HIPAA training. I have also had several years of experience in conducting research in a variety of contexts, and have always done so honestly and responsibly. As part of my training goals, I will attend the course, Responsible Conduct of Research and Related Policy Issues (G4010), at Columbia University Medical Center, in the first year, and the IRB Monthly Investigator Meeting, an on-site monthly seminar geared to ensure that research on human subjects is conducted in accordance with the federal regulations and in an ethical manner. Finally, I will attend, as relevant, seminars hosted by the Columbia University Center for Bioethics. Throughout my K01 award, I will be supervised by my mentor Dr. Weissman, who will advise and monitor me in the areas of conflict of interest, responsible authorship, data management and data sharing, as well as policies for handling misconduct and policies regarding research with human subjects.

Table 1 Timetable of Proposed Career Development: Didactics, Supervision, & Hands-on Practicum

Formal Didactic Training* **Supervision & Hands-On Practicum** **Seminars & Conferences**

Training Goals	Year 1	Year 2	Year 3	Year 4	Year 5
Goal 1: Training in phenomenology, assessment, and study designs of substance use and related behavioral disorders.	Epidemiology of Alcohol and Drugs **(P8470).** (2 hrs, weekly)	Perinatal Epidemiology (**P8422)** (2 hrs, weekly)			
	• Scheduled meetings with **Dr. Levin** (1 hr/Biweekly; first two years*); clinical phenomenology/study design • Scheduled meetings with **Dr. Hasin** (1 hr/Monthly; first two years*); assessments/instruments • Observership in the **Substance Treatment and Research Service (STARS**) (2 hrs/bi-weekly, first 6 months); • Participation in **Substance Abuse Fellows Seminar**, led by Dr. Levin (1 hr/ Weekly, as relevant)				
Goal 2. Training in Genetics, including (i) analysis of genetic data and (ii) integration of genetic and imaging data.	Methods in molecular/ Genetic Epidemiology **(P9407**) (2 hrs, weekly)	Neurobiology & Genetics of Psychiatric Disorders **(P8419**) (2 hrs, weekly)	Genetic Analysis Laboratory **(P8141**) (3 hrs, weekly)		
		Visit **Dr. Hamilton's** Laboratory at UCSF (~3 days)	Visit **Dr. Hariri's** laboratory at Duke University (~ 2 days)		
	• Scheduled conference call with **Dr Hamilton** (1 hr / biweekly)- genotyping methods/;identifying polymorphisms • Conference call with **Dr Hariri** (~1 hr / quarterly) – integration of genetic and imaging data • As needed consulting with **Dr. Hodge** – statistical analysis of genetic data • Twice yearly conference calls with **Dr. Weissman** and all mentors and consultants				
Goal 3: Training in use of longitudinal epidemiological and healthcare datasets relevant to smoking and substance use		Use of Large Scale National Health Care Datasets (**P6781)** (3 hrs, weekly)	Analysis of Repeated Measurements **(P8157)** (2 hrs, weekly)		
	• Scheduled meeting with **Dr. Brown** (1 hr/ monthly); birth cohort studies; prenatal exposures (also Goal 1). • Monthly meetings, **Sackler Institute for Development Psychobiology** (~2 hrs / month)				
ETHICS: Continued training in the Responsible conduct of Research	Responsible Conduct of Research and Related Policy Issues **(G4010)** (1 hr, weekly)				
	• IRB Monthly Investigator Meetings (1 hr / monthly) • Columbia University Center for Bioethics- Weekly Seminars (Attendance based on relevancy) • Overall supervision by **Dr. Weissman**				
SEMINARS & CONFERENCES (encompass all goals)	Weekly Seminars • New York State Psychiatric Institute / Columbia University Department of Psychiatry, Grand Rounds (1.5 hrs, weekly) • Department of Child Psychiatry Grand Rounds (1.5 hrs, attendance to be based on relevancy of topic) • Department of Biostatistics Journal Club (1.5 hrs, attendance to be based on relevancy of topic) Conferences • Annual attendance at the **American College of Neuropsychopharmacology** (ACNP) meeting • Alternating annual attendance at **World Congress of Psychiatric Genetics** & **College of Prevention of Drug Dependence (COPD)**				

*Then, on an as-needed basis.
All formal coursework (blue) is at Columbia University, and is one semester long

Table 2 Projected 5-year Distribution of Percent Effort on Research, Education, and Career Development.

ACTIVITY	Year 1	Year 2	Year 3	Year 4	Year 5
Execution of Research Plan	70	60	50	50	40
Coursework & Seminars	15	15	15	5	5
Mentorship Activities	15	15	15	15	15
Publications	0	10	10	10	20
Grants	0	0	10	20	20

A. SPECIFIC AIMS

The overall goal of this Mentored Research Scientist Development Award (K01) Application is to become an expert in applying translational methods to study the etiology of substance use disorders, by identifying genetic and neurobiological pathways that mediate individual risk for substance use and its related behavioral psychopathology. Exposure to prenatal smoking provides an excellent model with which to obtain these skills, while simultaneously tackling a serious public health problem. Despite the overall declining trends, smoking during pregnancy remains a leading cause of preventable illness among both mothers and offspring. Offspring of mothers who smoke during pregnancy are at heightened risk for a range of neonatal, as well as long-term adverse behavioral and substance use outcomes. Even though mothers who smoke while pregnant are likely to continue smoking during other times in the child's life, at least some of the above associations appear specific to prenatal exposure, suggesting an underlying biological mechanism. In the proposed research plan, I will examine how individual genetic and neurobiological variations contribute to the association between prenatal smoking exposure and offspring externalizing and substance use disorders. To do this, I will first examine functionally relevant genetic variations within monoamine oxidase A (*MAOA*) (an enzyme that deaminates serotonin *in utero*, and can be inhibited by a number of tobacco ingredients), as well as other exploratory genes related to the nicotine and dopamine family. I will then examine brain circuits implicated in self-regulatory behaviors, and in the final stage, I will integrate genetic and imaging hypotheses to explore how genes, exposures, and brain circuits interact on the pathway to risk.

My proposal will be developed on top of a longitudinal three-generation cohort study that has been prospectively followed for up to 25 years on a range of diagnostic measures, and now further includes genetic (DNA) markers and MRI scans. As the study was not originally designed to address substance use, and only limited information was acquired, I will implement an additional assessment wave that will add both diagnostic measures related to smoking, drug and alcohol use and behavioral measures of impulsivity and aggression. This will allow me to follow subjects prospectively through the age of risk on outcomes of interest, while at the same time taking advantage of 25 years of longitudinal assessments and biomarkers that I would not otherwise have been able to collect through the course of an MRSDA award period.

Few studies have examined the role of genes in moderating outcomes of prenatal exposures, and none with further indices of brain function. If funded, this study would be the first to directly test the role of genetic variation and cortical behavioral circuitry in the context of risks conferred by prenatal exposures. The execution of this research plan, in conjunction with the training aims detailed in the Candidate Section, will enable me to test biological pathways mediating adverse behavioral outcomes; to identify offspring groups who may be at greatest risk for these outcomes (and may require earlier or more targeted intervention); and ultimately, to export these skills to study other exposures, genes and behavioral outcomes relevant to substance use and its disorders.

Specific aims are as follows:

1. To test the relationship between prenatal exposure to maternal smoking and long-term offspring behavioral and substance use disorders. **Hypothesis 1A:** After adjusting for age and length of follow-up, offspring of mothers who smoke during pregnancy, as compared to those who do not (hereon, "exposed offspring"), will have higher rates of externalizing problems & disorders, including: (i) disruptive behavior disorders (conduct disorder and attention deficit hyperactivity disorder), and substance use disorders (including drug, alcohol and smoking); and (ii) dimensional behavioral trait measures of impulsivity and aggression. **1B:** Among the offspring with externalizing disorders, prenatal smoking exposure will be associated with an earlier age of onset.

2. To test whether genetic variation that alters fetal MAOA enzymatic levels increases the risk to offspring conferred by *in utero* exposure to maternal smoking. **Hypothesis 2A:** Offspring with the low expression variants of *MAOA* (*MAOA*-L) will have greater externalizing problems (as defined above) than those with high expression variants (*MAOA*-H). **2B:** MAOA genotype will moderate the association between prenatal smoking and offspring outcomes, with the exposed offspring having MAOA-L genotypes having the highest rates of externalizing problems.

3. To test whether brain circuits involved in behavioral inhibition mediate the relationship between prenatal smoking and offspring externalizing problems. **Hypothesis 3A**: Exposed offspring will perform more poorly (as indexed by greater errors, and shorter reaction times) on a task indexing self-regulatory behaviors (the Simon Spatial Incompatibility Task). **3B**: Exposed offspring will have reduced activation within cortical

regions involved in behavioral inhibition (particularly the anterior cingulate cortex [ACC], dorsolateral prefrontal cortex [DLPFC] and orbitofrontal cortex [OFC]) during the performance of the Simon Task, as well as reduced functional coupling between the cortical regions and the (i) striatum and (ii) amygdala. **3C**: Differences in performance (3A) and cortical activation (3B) will mediate the relationship between prenatal smoking and offspring externalizing problems.

4. To integrate imaging and genetic data, and examine whether genetic and imaging markers are together more predictive of offspring risk than either marker alone (exploratory). **Hypothesis 4A**: *MAOA* genotype will moderate the association between prenatal smoking and cortical activation during the behavioral inhibition task. **4B**: MAOA genotype will moderate the association between cortical activation and behavioral outcomes. **4C**: Offspring who are (i) exposed to prenatal smoking, (ii) have *MAOA-L*, and (iii) reduced cortical activity, will have the greatest/most severe externalizing problems.

I will also use a parallel hypothesis structure to examine variation within other genes of interest to substance use behavioral psychopathology, beginning with the nicotinic (especially the cholinergic nicotinic receptor subunit (CHRN) genes[30]) and dopaminergic (dopamine receptors *DRD2, 3 & 4*, the dopamine transporter, and catechol-*O*-Methyl Transferase[26, 31-33]) families. Both families of genes are also vulnerable to the effects of prenatal smoking exposure. Though the primary hypotheses are anchored around MAOA, examination of additional variants will allow me to target a broader range of behaviorally relevant genetic variation, to obtain additional fluency in genetic methodology, and provide pilot data for future grant applications.

B. BACKGROUND & SIGNIFICANCE.

B1. Significance Despite a gradual decline in overall rates of tobacco smoking in recent decades[34, 35], smoking remains prevalent today[36]. Smoking by mothers while pregnant is particularly worrisome[37]as it impacts both maternal and fetal health, and has been singled out as a leading cause of *preventable* illness among pregnant mothers and infants[35, 38]. The risks to the offspring are many, and begin *in utero*, with exposed offspring being at higher risk for premature and still births, physiological defects, and reduced birth-weight[39-45]. The risks continue through childhood in the form of cognitive and behavioral impairments[46-51], and ultimately, in adulthood, substance use and its associated disorders[51-55]. It is estimated that maternal smoking during pregnancy may account for a quarter of all externalizing behaviors[51]**.** Interestingly, offspring exposed to maternal post-natal smoking, but not pre-natal smoking, do not display the same adverse trajectories[56, 57], suggesting a biologically driven transmission.

Because tobacco is pharmacologically heterogeneous, mechanisms underlying the consequences to its exposure are likely complex. A number of tobacco ingredients (including invasive ones such as nicotine and carbon monoxide) not only traverse the placenta, but can reach even higher levels in the fetal compartment than in the mother following chronic exposure[37, 58]. Nicotinic receptors are located ubiquitously in the fetal brain, including on serotonergic and dopaminergic neurons where they serve a modulatory role. Serotonin (5HT) plays a critical role in neuronal growth, differentiation, and synaptogenesis during fetal development, laying down the foundations for cortical-subcortical connections that will ultimately mature into behavioral regulatory circuits. Upregulation by nicotine during key stages of fetal development may upset the required serotonergic balance, leading to impaired circuit formations in ways that predispose the fetus to problems of behavioral regulation (such as aggression or anti-sociality) that may not be detected until many years later[46, 59, 60]. A number of other tobacco ingredients can also alter 5HT balance pharmacologically via inhibition of monoamine oxidase A (*MAOA*)[61], thereby potentially resulting in synergistic effects on serotonin transmission transmission[62], as illustrated in **Figure 1**. I will test how variation in MAOA and other genes of relevance to externalizing psychopathology, as well as their ensuing neurobehavioral circuits, influence the risks of long term offspring behavioral problems and psychopathology resulting from prenatal exposure to maternal smoking.

B2. Background

Prenatal Smoke Exposure Interferes with Genetic Architecture. *In utero* exposures cannot change the fetus' existing DNA, but they can interact with existing genetic pathways to alter risk for subsequent developmental and long-term outcomes[63]. In recent years, a number of genes or genetic regions have been implicated in substance use, some of which are substance specific based on *pharmacological* pathways (e.g., alcohol dehydrogenase genes in alcohol dependence), and others predisposing to more common underlying *behavioral* correlates of substance use[64]. It is on the latter that this study focuses. Although I will examine additional nicotinic and dopaminergic variants, the primary target will be monoamine oxidase A (*MAOA*), a 15 exon long gene on chromosome Xp11.23[65, 66] that encodes the enzyme that deaminates amines, particularly serotonin[67, 68]. MAOA is particularly intriguing in the context of smoking pathways for a number of reasons: (1) the *MAOA* enzyme, is highly active *in utero*, rendering fetal serotonergic tone particularly vulnerable[69]. [MAOB, the other MAO enzyme variant, is not fully expressed until after birth]. (2) *MAOA* can be inhibited by a number of components of tobacco smoke (e.g., carbon monoxide, 2,3,6-trimethyl-1,4-naphthoquinone, farnesyl-acetone[61, 70]), providing a biological rationale to test mediation effects. These inhibitors can cross the placental barrier[71], and their action appears independent of nicotine, the primary addictive ingredient in tobacco[72]. (3) Genetic variation within *MAOA* has well-characterized functional effects. One region in particular, known as the untranslated variable number tandem repeat (VNTR) has been of much focus.

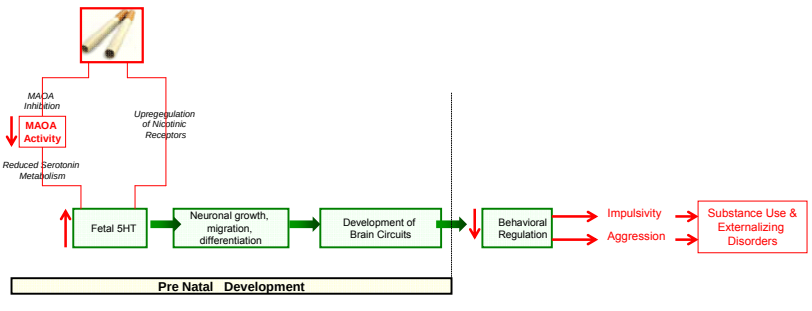

Figure 1: Hypothesized Pathways of Prenatal Smoke Exposure
Normal fetal development is shown in green. Red Arrows indicate how tobacco components might interfere with fetal development to predispose offspring to behavioral and substance use disorders.

The VNTR is a 30 base pair region within the promoter 1.2 kb upstream of the coding sequence that is present in repeat sequences of varying length[68]. The 3.5 and 4-repeat versions (known as the high functioning variant, "*MAOA*-H") are transcribed from two to ten fold greater than the 3 (and possibly 5) copy repeats (low functioning "*MAOA*-L")[68]. Although I will examine 8 other single nucleotide polymorphisms which together tag >90% of the known common variation in MAOA gene in Caucasian populations, the primary hypotheses will be anchored around this VNTR. Finally, (4) several lines of evidence converge on a genetic role for *MAOA* in the types of behaviors frequently seen among offspring exposed to maternal prenatal smoking. The pioneering work came from Brunner and colleagues[73] who showed that a mutation prematurely terminating a codon within the *MAOA* gene (thereby rendering hemizygous males functional equivalents of knockouts) led to higher rates of aggressive outbursts and violent impulsive behaviors[73]. Much animal and human research has since extended these observations. Mice with *MAOA* gene deletions show build up of pre- and post-natal serotonin in the hippocampus, frontal cortex and dorsal raphe nucleus[74], and exhibit aggressive behaviors which are reversible if *MAOA* expression is restored[75, 76]. Pharmacological inhibition of *MAOA* similarly increases aggression and craving[77, 78]. Humans with low-functioning *MAOA* variants demonstrate heightened impulsivity, aggression and sensation seeking behaviors, and are more likely to have drug and alcohol abuse[67, 79]. Finally, a number of studies have reported that the MAOA gene can moderate the effect of childhood maltreatment on an array of adult antisocial behaviors, with the findings overwhelmingly more robust among males (see meta-analysis[80]), and even detected in primates [81].

These collective observations provide a strong rationale to test how individual variation related to *MAOA* may contribute to the association between prenatal exposures and offspring externalizing psychopathology. This is a ripe question, as discussed in a recent review by investigators at the National Institute of Drug Abuse*: "Is fetal brain monoamine oxidase inhibition the missing link between maternal smoking and conduct disorders?* [61]" Only one study to my knowledge has directly examined *MAOA* in the context of prenatal smoking together. The authors of that study reported that *MAOA* uVNTR and maternal smoking interacted to predict childhood antisocial behavior[82], but did so differently among males (where *MAOA*-L was associated with increased risk) and females (*MAOA*-H was the risk variant). However, offspring in the study were still young (mean age ~15), and only conduct symptoms were tested. My proposal builds upon both this study by testing a range of behavioral and substance use psychopathology, and further examining the roles of brain circuitry, which I now turn to a discussion of.

Prenatal Smoke Exposure can Impair Development of Brain Structure and Function. Indices of brain structure and function, such as those provided by imaging techniques, allow us to examine whether the observed effects of genetic variation or exposures on psychopathology may be mediated by changes in brain morphology and functional circuitry. By doing so, they not only add explanatory power to our understanding of how genes may contribute to disease risk, and may further provide important intermediate phenotypes, which, by being more proximally allied to the underlying genetic architecture than diagnostic outcomes, may be easier to detect[83, 84]. Consider this in the context of serotonin and brain development, where serotonergic tone during fetal development is vital to proper neurogenesis[46, 59, 77, 78, 85-87]. Disruptions to this tone (whether genetically predisposed, induced by *in utero* exposures, or otherwise) may impair development of brain circuits (intermediary phenotypes), and the behavioral problems observed later in life might be mediated by this impairment.

MAOA Variation affects Brain Structure and Function. There is evidence that MAOA variation may alter brain structure, with the low functioning variant associated with structural loss in the anterior cingulate and orbitofrontal cortices, as well as the amygdala, insula and hypothalamus[24]. Reduced activation of cingulate and prefrontal cortices in response to conflict resolution and behavioral inhibition tasks have also been reported among low-expression MAOA carriers[24, 88, 89]. Few studies have directly examined brain changes resulting from *in utero* smoke exposure. One, the Saguenay Youth study, found reduced cortical thickness in the orbitofrontal, middle frontal, anterior cingulate, and parahippocampal gyrii, among exposed offspring [90], but did not test whether these changes might mediate the relationship between maternal smoking and the offspring outcomes. My proposed study will be the first to directly test whether the offspring risk of long-term externalizing psychopathology resulting from prenatal smoking exposure is mediated by impaired brain circuits.

Externalizing Psychopathology and Brain Function. Externalizing behaviors are thought to arise in part from a failure of "top-down" regulation— that is, failure of frontal cortical structures to exert appropriate inhibitory control over activity within sub-cortical structures, especially the limbic and para-limbic regions [91-95]. Over the last decade, advances in imaging techniques have allowed these hypotheses to be directly tested in the working human brain. For example, a recent MRI study reported that psychiatric patients had smaller grey

matter volumes within the orbitofrontal cortex, which predicted aggression ratings[96]. Another study of bipolar patients found reduced activation within cingulate and temporal regions when performing a response inhibition task[97]. Anterior cingulate volumes were also found to correlate with impulsivity ratings among patients with borderline personality disorder[98]. Finally, a study of healthy adolescent boys reported that volume within the ventromedial prefrontal cortex was inversely correlated with both parent- and teacher-reported impulse control measures[99]. Similar regions have been implicated in studies of conduct disorder, which report cingulate deactivation in response to negatively valenced stimuli[100, 101], and blunted event related potentials in the left prefrontal cortex during memory tasks[102]. Finally, the anterior cingulate, prefrontal, and orbitofrontal regions have all been implicated in addictive disorders, with volume loss[103, 104], lower glucose metabolism [105-107], and task specific hypoactivity[33, 108, 109] reported among both alcohol and drug dependent subjects. Interpretation of imaging studies of substance use disorders however requires additional caution, as different phases of the disorders may invoke different neurobiological profiles[110] and chronic drug use (including smoking) can itself lead to structural or functional brain changes[22, 111]. I will address this both by examining behavioral traits that may serve as antecedents of substance use disorders, and also by longitudinally following subsets of individuals with no substance use history at the time of the brain scan, which will allow me to tease out predisposing from compensatory brain changes.

Based on data supporting a role in executive control, vulnerability to *MAOA* variation, and structural changes following nicotine exposure, I will focus primarily on the anterior cingulate cortex (ACC), as well as the dorsolateral prefrontal cortex (DLPFC) and orbitofrontal cortex (OFC). The ACC is particularly noteworthy in the context of prenatal smoking, as it is densely innervated by serotonergic input from the dorsal raphe nucleus, and has the single highest density of serotonergic receptors of any cortical region[112], making it particularly vulnerable *in utero* to variation in serotonergic tone resulting from smoke exposure. Behavioral response inhibition will be assessed using the Simon Spatial Incompatibility Task[113] (detailed in section D4 of the Methods), an analog of the Stroop Word-Color Interference task[114] that requires subjects to inhibit a more naturalistic or automatic behavioral response in favor of less automatic one. Inhibiting the prepotent reading response in this condition requires the mobilization of attentional resources, the resolution of cognitive conflict, modulation of the automatic response tendency and, engagement of regulatory control. Previous studies have revealed significantly greater anterior cingulate and prefrontal activation during the high conflict components of the task (requiring the greatest regulatory control), in both adults[44, 115-119] and children[120, 121]. Individuals who are impulsive, in that they act without foresight or planning, typically perform poorly on measures of inhibitory control such as the Simon and Stroop tasks, and may have reduced frontal activation during the high conflict component, reflecting an impairment in self-regulatory capacity. In addition to examining this frontal impairment, I will also examine functional coupling between the frontal regions and two sub-cortical regions, the amygdala and striatum, in order to test whether disruptions in regulatory circuits contribute to the risk for offspring psychopathology. The frontal cortex, and particularly the ACC, potently inhibits the amygdala, forming the cortico-amygdalar regulatory circuit[122], and lesions of this circuit lead to emotional dysregulation and increased aggression[123]. Cortico-striatal circuits regulate impulse control and reward dependent behaviors[124], with circuits to the dorsal striatum preferentially implicated in response inhibition, and to the ventral striatum predominantly regulating delay aversion[125].

Integrating Genetic and Neurobiological Measures will better target mechanisms of psychopathology. In the final stage of the project, I will merge genetic and neurobiological data to examine how the different risk factors assessed in the study may interplay to predict ultimate risk of offspring psychopathology. For example, one might predict that offspring who have the highest loading of risk (i.e., having a mother who smoked, having the low functioning *MAOA* variant, and the least cortical activity or cortical regulation) will have the highest rates of externalizing problems, those with the lowest risk (nonsmoking mother, high functioning *MAOA* and no cortical reduction), the lowest risk, and other combinations in the middle. This has never been tested before, and though exploratory, this analysis will allow me both (1) to test cumulative models of risk leading to disorders of interest, and (2) to identify offspring groups at highest risks for these disorders, which may have eventual implications for intervention strategies.

C. PRELIMINARY STUDIES

Data presented in sections C1 through C4 were conducted as part of grant MH36197(Weissman, P.I). Data in C1-C3 are being prepared for publication (Talati et al., first author). They have been presented by me at the 2008 American College of Neuropscyhopharmacology annual meeting, and were the recipient of a 2009 Early Career Investigator Award from the International Society for Psychiatric Genetics. Childhood behavioral data (Fig. 2) and imaging data (Fig. 4) were analyzed for this application and have not been previously presented.

C1. Prenatal smoking is associated with offspring conduct, drug and alcohol use disorders, and with childhood measures of impulsivity and aggression. In order to test for genetic and neurobiological underpinnings of the effects of prenatal exposure, I first document that there is an epidemiologically robust association. **Table 3** shows lifetime rates of drug, alcohol, and psychiatric disorders among offspring, based on whether or not they were exposed to maternal prenatal smoking. After adjusting for cumulative length of follow-up (using survival methods) and proband depression, female offspring exposed to maternal prenatal smoking, as compared to those who were not, were at a three fold increase for both drug use disorder (34 % vs 10%; Adjusted Hazard Ratio = 3.2 [95% CI: 1.2, 8.5], p = .02) and alcohol use disorder (31% vs 10%, HR = 3.2 [1.2, 7.2], p = .02). When subset into abuse versus dependence, patterns appeared similar for instance, 22% exposed vs. 7% unexposed offspring had some dependence, and 22% vs 9% had some abuse]. No associations with substance use disorders were observed in male offspring, possibly reflecting the higher overall rates among the unexposed males which precluded any additional impact). Exposed male offspring were instead at more than two-fold increased risk for conduct disorder (52 vs 28%, HR = 2.4 [1.1, 5.5] p = .04). Formal gender by exposure interaction tests further revealed significantly different associations between male and female gender for drug (p = .05) and alcohol (p = .01) use disorders. Finally, preliminary analyses of childhood behavioral symptom data also showed that exposed male offspring had higher overall externalizing (but not internalizing) symptoms, as well as increased aggression, rule-breaking, and impulsivity scores **(Figure 2).** Female offspring did not differ by exposure on any of these measures.

	Female Offspring			Male Offspring		
	Prenatal Smoke Exposure			Prenatal Smoke Exposure		
	NO N = 64	**YES** N = 29	**HR*(95% CI), *p***	**NO** N = 54	**YES** N = 19	**HR* (95% CI), *p***
Any SUD+	12 (18)	13 (44)	**2.4 (1.1, 5.2), .02**	26 (50)	9(47)	.7 (.4, 1.6), .48
DUD+	7 (10)	10 (35)	**3.2 (1.2, 8.5), .02**	13 (25)	4 (21)	.7 (.3, 2.0), .52
AUD+	7 (10)	9 (31)	**2.9 (1.2, 7.2), .03**	23 (44)	6 (31)	.4 (.1, 1.2), .11
Dependence+	6 (7)	8 (22)	2.8 (.9, 9.1), .07	17 (23)	4 (10)	.5 (.2, 2.1), .44
Abuse	8 (9)	78 (22)	2.3 (.9, 6.1), .09	22 (30)	9 (24)	.8 (.4, 1.9), .68
ANY DBD	13 (20)	8 (28)	1.4 (.6, 3.6), .47	18 (34)	10(52)	1.6 (.7, 3.5), .20
CD	13 (20)	6 (20)	1.0 (.3, 2.9), .90	15 (28)	10(52)	**2.4 (1.1, 5.5), .04**
ADHD	2 (3)	2 (7)	-	7 (13)	3 (16)	.9 (.2, 3.8), .78
MDD	35 (54)	15 (50)	.9 (.4, 1.8), .81	22 (42)	7 (26)	.7 (.4, 1.4), .39

Table 3. Rates of offspring psychopathology by prenatal smoke exposure
*HR: Hazard Ratio, adjusted for cumulative length of follow-up, and familial risk for depression, using proportional hazards models.
+: Categories for which there was a statistically significant (at p < .05) gender by exposure interaction.
AUD Alcohol Use Disorder; ADHD: Attention Deficit Hyperactivity Disorder: CD: Conduct Disorder; DBD: Disruptive Behavior Disorder; DUD: Drug Use Disorder; MDD: Major Depressive Disorder; SUD: Any Substance Use Disorder. Substance Use Disorders have been categorized both by substance and by disorder type. Thereby, AUD and DUD include both abuse and dependence categories. Conversely the abuse and dependence outcomes include both drug and alcohol use.

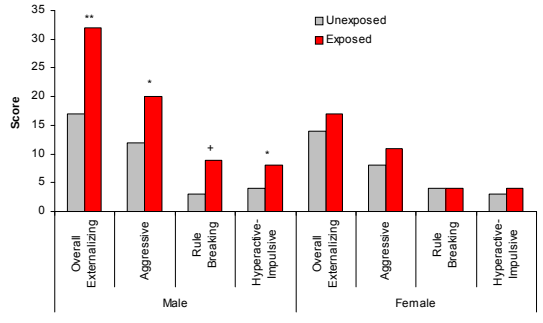

Fig.2: Childhood behavioral measures by prenatal smoke exposure. ** N < .01; * N < .05; + p < .01; adjusted for age and familial risk for depression. N = 41F/33M.

Accounting for confounding variables. An oft-cited concern with studies of prenatal smoking is that mothers who smoke during pregnancy are likely to be different from those who do not on a variety of other factors. We therefore attempted to account for as many demographic, pregnancy, and psychiatric history related variables as possible that could have been differentially associated with smoking mothers and hence confounded results.

There were no differences among smoking and non-smoking mothers, or among their respective offspring, by any demographic variables, including age, gender (of offspring), maternal or offspring education, household income, or religious denomination. We next examined whether other variables related to the mother's pregnancy might explain the association between prenatal smoking and the above outcomes. Neither the mother's nor father's age at the time the child was born, number

of prior pregnancies, or any types of complications during pregnancy or delivery were associated with the mother's smoking.

Exposed offspring were born at lower weight than unexposed (6.8 vs. 7.6 lbs, p = .002; although it is unclear whether this relatively small difference is clinically meaningful). Mothers who smoked during pregnancy were also more likely to consume any alcohol (55 vs 35%, χ^2= 8.3, p= .003) and daily caffeine (64 vs 11%, χ^2= 50, d.f. = 1, p < .0001), but not prescription medications. Neither mothers nor fathers differed by lifetime rates of substance use disorders or antisocial personality disorder (although we cannot rule out the effects of less pervasive or diagnosable antisocial behaviors). Because maternal alcohol and caffeine use, as well as offspring birth-weight, were associated with maternal smoking during pregnancy, we tested whether these variables could explain the relationship between maternal smoking and substance use disorders in girls, or between maternal smoking and conduct disorder in boys. However, none of the above associations were altered significantly when birth-weight or maternal alcohol or caffeine use were added as covariates in to the models, either individually or together. Finally, we have previously documented in this cohort that though maternal pre- and post-natal smoking were correlated (as might be expected), offspring outcomes could not be explained by post-natal exposure[49].

C2. Genetic Variation within the Serotonin 1B receptor is associated with conduct disorder and alcohol use disorder. Preliminary analyses focused on a subset of subjects and polymorphisms related to serotonin variation, identified a polymorphism, rs6298, located within the coding region of serotonin 1B receptor (HTR1B) that was associated with both conduct disorder (CD) and alcohol use disorder (AUD) (there were no associations observed for the serotonin transporter, including the well known promoter linked polymorphism (5HTTLPR). After formally adjusting for age of follow-up, gender, and proband depression, the presence of the "TT" genotype at rs6298 was associated with a more than five-fold increase in the risk for AUD, and nine-fold increase in the risk for CD (**Table 4).** When the associations were stratified by gender, the association between HTR1B genotype and offspring outcome appeared similar across male and female offspring: for example, 71% of males with TT, as compared to 8% of those with CC/ CT, had CD, and 25% of TT females, as compared to 10% of CC/CT females had CD. For AUD, 51% and 22% of males with and without the TT genotype respectively had AUD; 33% vs 14% of females with and without TT had AUD. Importantly, subjects who were gave blood and were genotyped did not vary significantly from those who did not, either by overall length of follow-up (26.0 vs. 25.0 yrs, p = .55) or, importantly, by exposure to maternal prenatal smoking (31% vs 24% exposed, p = .18).

Diagnosis		Genotype at rs6298 CC N (%)	CT N (%)	TT N(%)	HR (95% CI), *p*
SUD	*Yes*	24 (50)	18 (38)	6 (12)	2.4 (.9, 6.1), .07
	No	70 (50)	60 (43)	10 (7)	
DUD	*Yes*	15 (56)	10 (37)	2 (8)	.7(.1, 6.1), .79
	No	79 (49)	68 (42)	14 (8)	
AUD	*Yes*	15 (41)	16 (43)	6 (16)	**4.4 (1.4, 13.8), .003**
	No	79 (52)	62 (41)	10 (7)	
Depend.	*Yes*	9 (50)	6 (33)	3 (16)	3.7 (1.1, 12.9), .04
	No	45 (59)	29 (38)	3 (4)	
Abuse	*Yes*	14 (66)	14 (19)	3 (14)	2.6 (.9, 7.5), .08
	No	40 (54)	31 (42)	3 (4)	
DBD	*Yes*	14 (48)	10 (35)	5 (17)	**6.4 (2.3, 17.3), .0003**
	No	80 (50)	68 (43)	11 (7)	
CD	*Yes*	9 (45)	5 (25)	6 (30)	**9.4 (3.4, 25.6), <.0001**
	No	77 (55)	58 (41)	5 (4)	
ADD	*Yes*	4 (44)	4 (44)	1 (11)	2.3 (.4, 16.2), .37
	No	73 (58)	46 (36)	8 (6)	
MDD	*Yes*	27 (51)	21 (40)	5 (9)	1.4 (.6, 3.1), .21
	No	59 (55)	42 (39)	6 (6)	

Table 4. Rates of offspring psychopathology by HTR1B Genotype
Percentages are row-wise, i.e., denote the percentage of persons with or without the disorder who had a given genotype. Hazards Ratios (HR) compare the risk for each disorder conferred by having the TT genotype to that conferred by having either the CT or CC genotypes, after adjusting for length of follow-up, gender and familial risk for depression. (*See Table 3 for list of abbreviations and classifications of disorder categories*).

C3. Serotonin Receptor Gene Variation can moderate the association between prenatal exposure and offspring behavior Because both maternal prenatal smoking and serotonin 1B receptor genotype predicted offspring externalizing disorders individually, I tested whether the effects of prenatal smoking on offspring disorders might vary based on the genotype of the serotonin receptor genotype: in other words, did offspring HTR1B genotype moderate the effects of maternal prenatal exposure? To do this, we first examined the association between maternal smoking and offspring disorder for each genotype separately. Among offspring with one or more C alleles, there was no association between maternal smoking and conduct disorder: 8/83 (11%) unexposed, and five of 31 (16%) exposed, offspring developed CD (p = .33). However, among offspring with the "TT" genotype, none of the unexposed, but all five of the exposed offspring, developed CD (p =.005). The trajectories of each of these groups by onset age are outlined in **Figure 3.** Even

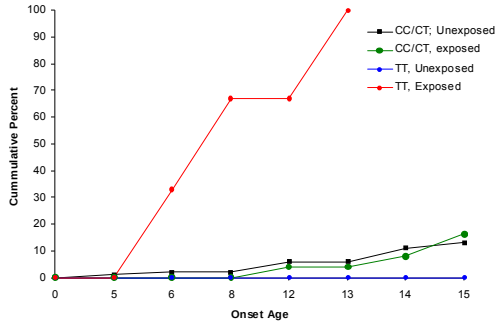

Figure 3: Cumulative onset of conduct disorder, stratified by exposure status and HTR1B genotype. Each data point reflects the percentage of offspring in each group who had an onset of CD by the corresponding age on the x axis. Gene-exposure interaction = .04.

though groups were small, comparison of the two distributions revealed a significant genotype by exposure interaction (χ^2= 5.2, d.f. = 1, *p*= .02), that remained statistically significant following: (1) analysis of the genotypes as a 3-class variable (2) analysis of allele counts rather than genotypes (3) random selection of only one individual per family, or (4) restricting the analysis to the second generation only. Because of the small sample size, and the low expected frequency of the risk-conferring TT genotypes (<10% in the Caucasian samples), we did not have the power to further adjust for potential confounding variables in this final analysis.

These findings are consistent with animal models showing that HTR1B receptor knock out mice have increased aggression, hyperactivity, and vulnerability to drug abuse, relative to their wild-type counterparts[126, 127] as well as with human mutations that have been associated with aggression [128], hostility [129], substance use[130], and ADHD[131]. Rs6298 lies within the coding region of HTR1B but is silent (i.e., does not alter the coded amino acid) and therefore unlikely to play a direct causal role. The proposed training in genetic methods will allow me to follow up on these findings and perform subsequent genotyping and mutation discovery in order to identify the causal variants.

C4. Prenatal Smoking is associated with reductions in cortical mantle that are independent of familial risk for depression. As part of the most recent (fifth) wave of the study, brain scans were collected on 216 members of this cohort. The first 131 individuals from the scan, ranging from Age 6-54, were originally analyzed by familial risk for depression (i.e., high versus low). The comparison revealed large expanses of thinning (with an average 28% reduction in thickness) across the lateral aspects of the parietal, posterior temporal, and frontal cortices of the right hemisphere in the high risk group[132]. Because the study had been originally designed to study depression, it was important to ascertain any differences ensuing from other risk factors would not be masked by the differences due to depression risk. We therefore examined changes in cortical structure resulting from exposure status, and tested whether these differences were conserved after adjusting for familial depression risk. As shown in **Figure 4**, prenatal exposure was associated with thinning of the cortical mantle in fronto-parietal regions as well as the cerebellum (both predominantly in the right hemisphere), but the thinning was more diffuse than and did not overlap with the changes correlated to depression risk. This is consistent with our clinical data where prenatal smoking was associated with conduct and substance use problems among but did not predict internalizing problems among offspring of either depressed or non-depressed parents. Though the functional data are not yet available for analysis, the above data demonstrate the ability to separate exposure related from depression risk and study design related brain changes.

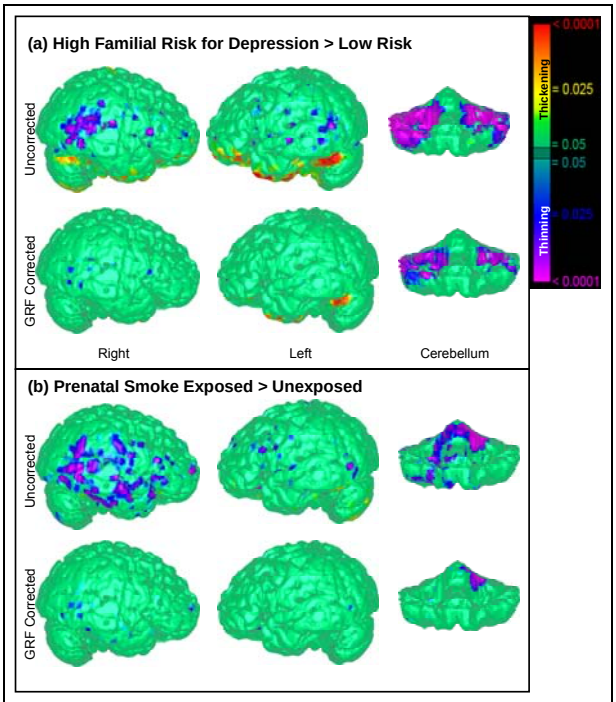

Figure 4. Demonstration of differential thinning of the cortical mantle and cerebellum based on familial risk for depression (upper panel) and prenatal exposure (lower). Purple areas indicate significantly reduced thickness. All analyses control for offspring age and gender; Analyses of prenatal smoke exposure are adjusted for risk status for depression. Data are shown both unadjusted (top) and corrected for multiple comparisons using Gaussian Random Field Theory (bottom). (*Data were collected as part of MH36197, Weissman, P.I; Peterson; Co-PI)*

D. RESEARCH DESIGN AND METHODS

D1. Sample This study builds upon an existing 3-generation longitudinal cohort. I provide first an overview of the sample; section D2 will then detail specific existing as well as proposed assessments. In the original study, probands (Generation 1) with major depression were selected from outpatient clinical specialty settings for the treatment of mood disorders in the New Haven, CT, area. Only subjects with moderate to severe MDD that resulted in impairment were selected. Non-depressed probands were selected concurrently from a sample of adults in the same community, and were required to have no lifetime history of psychiatric illness, based on several interviews. The sample was entirely Caucasian, and primarily of Italian ancestry. The study was initiated in 1982, and the sample has been followed for a total of five waves: wave 1 (year 0); wave 2 (year 2); wave 3 (year 10); wave 4 (year 20) and wave 5 (year 25). Probands (G1) and their offspring (G2) were followed from the beginning of the study; commencing with time 10, grandchildren (G3) were also directly interviewed. All study procedures for each wave were approved by the institutional review board at New York State Psychiatric Institute/Columbia University. Informed consent was obtained for all adults; for minors, assent was obtained, with written consent from their parents. Sample retention over time has been excellent, with at least 80% of the sample participating in 3 or more waves. The study has yielded more than 20 publications (e.g.[20, 21, 49]).

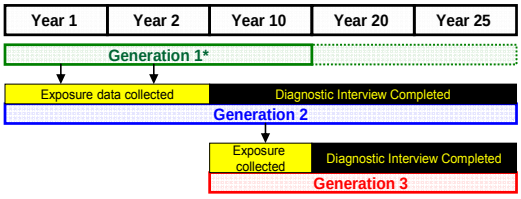

Figure 5: Study Design. Subjects included in the proposal had a maternal assessment of prenatal exposure in the assessment waves shown in yellow, followed by one or more interviews in the subsequent waves (black)

D2. Clinical Assessments. The battery of collected assessments is extensive, and is summarized in **Appendix 1.** I detail here the assessments relevant to my proposal, as well as new assessments I will include.

(a) Assessment of Smoking During Pregnancy Smoking by the first Generation mothers (G1) during pregnancy was assessed at Wave 1 or 2; for G2 mothers, smoking was assessed at Wave 3. (The average child age at the time of the mother's report was 11 years). I will include mother-child pairs for which there was at least one direct offspring interview following the wave at which maternal smoking was assessed (that is, at least two time points), as shown in **Figure 5.** Mothers completed a Medical Report for each pregnancy, which included questions about the course of pregnancy and delivery, as well as the child's post natal and early developmental milestones. [Questions about smoking were located midway through the report, so a response bias based on smoking behaviors is unlikely]. Mothers were asked whether they had smoked more than 10 cigarettes per day while pregnant, and if so, how often: 1-2 times, 3-5 times, 6-10 times, every two weeks, weekly, or daily or almost daily. The frequencies are shown in **Figure 6**, stratified to show that patterns are similar by generation. Given this distribution, I generated a dichotomous variable based on whether or not the mother reported smoking 10 or more cigarettes per day, almost every day, which will constitute the primary exposure variable. Even though the dichotomization was data-driven, this cut-off is commonly employed in the literature to define heavy smokers, and is consistent with data showing that exposure to ½ a pack daily is required for adverse offspring behavioral effects to be reported[133]. Analogous questions were asked for drug and alcohol use during pregnancy. Because no mothers reported drinking alcohol more than bi-weekly, the variable was encoded based on any use during pregnancy. Only four mothers reported illicit drug use during pregnancy, and these were removed *a priori* from the analysis. Eighty three percent of mothers completed the report (non-responders, notably, were mothers who did not complete the report, not those who did not complete the specific smoking question). Additionally, in 23 cases where the father also filled out a child report, there was 100% concordance between maternal and paternal reports of the mother's prenatal smoking.

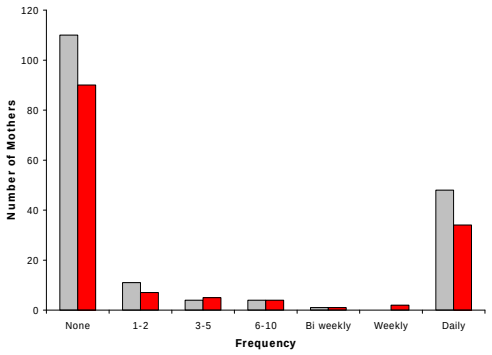

Figure 6: Frequency of Maternal Smoking during Pregnancy. Rates are shown separately for the second (grey) and third (red) generations.

(b) Diagnostic Interviews were conducted using a semi-structured diagnostic assessment (the Schedule for Affective Disorders and Schizophrenia–Lifetime Version for adults[134], and the child version modified for the DSM-IV for subjects between ages 6 and 17 years)[134] (At wave 4, the Schedule for Affective Disorders and Schizophrenia–Present and Lifetime Version for Children was used[135]). For all subjects, the first interview

always assessed the entire life period up to that point; follow-up interviews assessed the period since the preceding interview. Diagnoses are therefore cumulative, and the age at last interview reflects years of follow up. Assessments were administered by trained doctoral- and masters-level mental health professionals, blind to previous assessments of the same subject, smoking status of the mother, and clinical status of other family members. Final diagnoses were made using the best-estimate procedure [136] by two experienced clinicians who were also blind to smoking, diagnostic or family variables. Inter-rater reliability scores, both across interviewers and across best estimators, were high[49].

(c) Childhood Behavioral Measures. I selected *a priori* three scales of the Child Behavior Checklist (CBCL)[137]: Aggressive Behavior Scale (AB), Rule-Breaking (delinquent) Behavior Scale (RBB), and the Hyperactivity-Impulsivity sub-scale of the Attention Deficit Subscale (HI). The AB and RBB are empirically derived based on symptom clustering; the HI is based on DSM categories. The CBCL instrument is intended for subjects aged 7-17. Each symptom is assigned a score from ranging from 0 to 2 by the child's mother, based on how truly or frequently she ascribes the symptom to her child. Scores for each item are summated to generate a total score. Even though reported by the mother and not clinician related, there is substantial correlation with DSM disorders; specifically, AG scales correlated .64 with DSM Oppositional Defiant Disorder, and RBB scales .63 with Conduct Disorder; overall externalizing scales correlate .62 with CD [137].

(d) Proposed Follow-Up Schedule. The 346 subjects in the study with data on prenatal smoke exposure (**Table 5**) will be targeted for follow-up. Assuming a 75% retention rate (this is a very reasonable estimate, as the overall sample retention over the years has been ~80%) will yield N = 259 (All 259 subjects will be 18 by the fourth year of the award period; no subjects will be contacted while still minors). Subjects will be contacted first by mail, and then by telephone, and invited to participate in a telephone interview. Those who provide informed consent will be contacted by a trained clinical interviewer, and undergo a SADS-L update interview, which will include assessment of drug use disorders and alcohol use disorder from the time since last interview, a lifetime assessment of nicotine dependence (which was never assessed previously), and assessment of depressive and anxiety disorders since the last interview. I have chosen to use the SADS to ensure instrumental consistency, as this was used at previous waves of the study. However, previous studies have shown that for alcohol and drug dependence, diagnoses appear robust across instruments[138].

Four additional behavioral measures will be administered, selected based on their relevance to behavioral dimensions of substance use, as well as for brevity, to avoid subject fatigue and the lower validity associated with overly long questionnaires[139] .Together, the four questionnaires should take ~15 minutes to administer. (i) The Barratt Impulsiveness Scale-11 (BIS-11)[140] is one of the most frequently used measures of trait impulsiveness. It includes 30 questions, scored on a scale from 0-4, that yield a total score, and 6 first order (attention, motor, self-control, cognitive complexity, perseverance, and cognitive instability), and 3 second order (attentional, motor and non-planning) factors. A total score of 72 or higher typically classifies an individual as highly impulsive. Internal consistency is high, with coefficients ranging from .79 to .83 for undergraduate, substance abusing, and general psychiatric patients, and the scale has good reliability and validity with other measures of impulsiveness[141]. BIS-11 has not only differentiated users from non-users, but also early- from late-onset alcoholics, violent from non-violent criminal offenders, and among smokers, the number of cigarettes smoked (reviewed in [142]). Finally, the scale has been used in genetic studies, with one study[143], but not another[89] reporting a negative relationship between MAOA activity and BIS-11. (ii) the impulsive sensation seeking dimension of the Zuckerman-Kuhlman Personality Questionnaire Version III-R (ZKPQ-III-R)[144-146]. The dimension includes 19 true/false items. Reliability coefficients of the ZKPQ-III-R are high (> .8) and the scale measures constructs similar to other both shorter and longer sensation seeking instruments[147]. Importantly, the trait also correlates inversely with MAO levels[146]. (iii) The Buss Perry Aggression Questionnaire (AQ) [148, 149]is an updated version of the Buss-Durkee Hostility Index. It includes 29 questions, scored on a scale from 1 to 5, that are grouped into four factors: physical aggression, verbal aggression, anger and hostility. The AQ has high internal consistency[148] been validated in both college students and in population-based samples. (iv) The Faegerstrom Test for Nicotine Dependence (FTND)[150] is a brief 6 question report of smoking behaviors, that is rated on a scale from 0 (low dependence) to 10 (high). Scores greater than 5 suggest a moderate level, and over 8, a high level of dependence. Internal consistency for the FTND is .61, and the scale is closely related to biochemical indices of heaviness of smoking including carbon monoxide exhalation and salivary nicotine and cotinine[150].

D3. Genotyping methods: *MAOA*-VNTR is a common putatively functional variant of *MAOA* [151]. Genotyping procedures for the VNTR region will be as follows: polymerase chain reaction (PCR) amplification of the VNTR in the upstream regulatory region of *MAOA* will be carried out using the following primers: 5'-

acagcctgaccgtggagaag-3' and 3'-gaacggacggctccattcgga-5', with one primer labeled with a fluorescent dye. Amplification is performed in a final volume of 10 µl containing 20 ng of genomic DNA template, 50 µM dNTPs, 1 M anhydrous betaine, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, and 0.5 U Platinum *Taq* DNA polymerase. Samples are denatured at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final 10 min step at 72°C[152]. PCR products will be separated on an ABI Prism 3700 DNA Analyzer and alleles will be scored using Gene Mapper software (Applied Biosystems). The genotyping procedures have been previously published [151]. Three and 5 copy variants will be scored as low expression MAOA, and 3.5 and 4 as high functioning. However, because the 3.5 and 5 variants are rare, particularly in Caucasian populations[68], I expect almost all subjects to have 3 or 4 copies.

For single nucleotide polymorphism analysis, HapMap data targeting MAOA was used to choose markers with minor allele frequencies greater than 0.05, and pair-wise $r^2 \geq 0.80$ in the CEU HapMap samples as a way to maximize the coverage of the region. The following eight polymorphisms were selected: rs6323, rs2283724, rs1800464, rs3027415, rs12843533, rs6609257, rs1181275 and rs2179098, which together cover 96% of common variants using standard tagging algorithms. SNPs will be genotyped using Taqman technology (Applied Biosystems, Foster City, CA). Assays will be made as follows in a 5µl reaction volume: 10ng/µl DNA dried down, 1X Applied Biosystems Taqman Universal PCR MasterMix, 0.5X Taqman assay mix. The reaction will be cycled for 1 cycle of 95ºC for 10 mins, and 50 cycles of 92ºC for 15 sec, and 60ºC for 60 sec on an Applied Biosystems GeneAmp PCR System 9700. Genotypes will be called using SDS 2.1 software. All polymorphism data will be filtered for Mendelian segregation, and families with errors will be regenotyped and unresolved errors will lead to the family being dropped from analysis. Only variants with call rates >95% will be used for analysis. A subset of 10% of samples will be genotyped in duplicate.

D4. Imaging methods. Scans have been acquired on 214 subjects. Functional imaging data will be analyzed by different investigators for studies relating to depression; I will analyze the imaging data in relation to prenatal exposures and substance use outcomes.

(a) The fMRI Simon Spatial Incompatibility Task A series of white arrows pointing either left or right were displayed against a black background either to the left or right of a white gaze fixation cross-hair positioned at midline. The majority of stimuli were congruent; i.e. arrows pointing in the same direction as their position on the screen; e.g., a rightward-pointing arrow presented to the right of midline). A smaller number of stimuli were incongruent; i.e., pointed in a direction opposite their position on the screen; e.g. a left-pointing arrow presented to the right of midline). Subjects were instructed to respond as quickly as possible to the direction of the arrow by pressing a button on a response box using the index finger of their right hand for a left-pointing arrow and the middle finger of that hand for a right-pointing arrow. Subject responses and reaction times were recorded for each trial. Stimulus duration was 1300 msec, with an interstimulus interval of 350 msec; each run was 102 stimuli (2 min 48 sec) long, with 7 incongruent stimuli presented pseudo-randomly every 13-16 congruent stimuli (i.e., 21.46 - 26.4 sec apart). In each run, 51 arrows each pointed left and right; and 51 each to the left and right of midline. Stimuli were presented against a black background and back-projected onto a screen positioned in front of the subject at the opening of the magnet bore. Because the only difference between conditions is the congruence or incongruence of the task-relevant and -irrelevant features of the task, this model fully controls for the physical features of the stimuli, and the resultant fMRI signal can be attributed to neural components that resolve the interference that the presence of the task-irrelevant features produces.

(b) fMRI Acquisitions Images were acquired on a GE Signa 1.5 Tesla scanner equipped with an echoplanar imaging system (Milwaukee, WI), a standard quadrature head coil, and a T2*-sensitive gradient-recalled single shot echo planar pulse sequence. T1-weighted axial-oblique slices were acquired parallel to the anterior-posterior commissure (AC-PC) line; functional images were acquired using a gradient-recalled single shot echo planar pulse sequence, at the same locations as the 10 axial T_1-weighted slices, in runs of 1020 images, or 102 per slice. Repetition Time (TR) = 1650 ms, Echo Time (TE) = 60 ms, flip angle = 60°, acquisition matrix 128 x 64, field of view = 40 x 20 cm, slice thickness=7 mm, in-plane resolution of 3.12 x 3.12 mm.

(c) fMRI Analyses Images will be analyzed using SPM5 (Wellcome Group, UK). Prior to analysis, fMRI will first be visually inspected for artifacts, and then motion corrected using SPM5 software with realignment to the middle image of the middle run. Images are discarded if the peak motion estimates from SPM exceed 1mm displacement or 2 degrees rotation[153]. Drift of baseline image intensity is removed using a high-pass filter based on discrete cosine transform (DCT) with a cutoff of frequency of 1/128Hz. Low intensity pixels outside of the brain are removed, and the images are spatially smoothed using a Gaussian filter with a full width at half maximum of 6.3 mm. The T_1-weighted axial anatomical images and corresponding echo-planar functional images for each subject will be transformed into a common Talairach space[154]. To account for hemodynamic

lag, the first two images (representing 3.3 sec) in each time series will be excluded. The voxel-wise change in fMRI signal will then be calculated for each subject using a General linear model that extracts signal differences (t-contrasts) across the different conditions (i.e., incongruent with correct answers (IC), congruent with correct answers (CC), incongruent with incorrect answers (II), and congruent with incorrect answers (CI)). The coefficients for the adjusted signal differences between the IC and CC conditions will form the primary dependent variable in each ROI. Random field theory based methods will be used to adjust the threshold of p-value for measuring significant task-related brain activity. The ROI for the cingulate and other frontal cortical regions, as well as the amygdala will be defined using standard stereotactic coordinates[155]; those for striatal regions will be hand circumscribed on the T1-weighted axial images by a trained neuroanatomist. Sub-regions within the ROIs will also be examined.

Functional connectivity between regions will be assessed using coherence analysis[156, 157], which measures the coupling of BOLD signals among different brain regions. The method is more sensitive than covariance measures in that it accounts for regional differences in hemodynamic response functions, which can be substantial[158]. Briefly, the time course of voxels with the 10 highest t-contrast values within the seed region are averaged (though other criteria can also be used), and coherence coefficients between the seed and each voxel in the region for which connectivity is being tested are computed. Though correlative in nature, measures such as coherence likely index physiologically relevant functional connectivity between spatially discrete brain regions[159]. I will also compare the functional connectivity that is driven entirely by the task from that which is not, by estimating the partial coherence[156] between the regions after accounting for the relationship of each region to a reference series modeling the stimulus presentation. Finally, I will explore coherence between the seed region and all voxels in the brain.

D5. Data Analyses The sample sizes for the analyses described in this proposal are detailed in **Table 5,** which includes breakdown by gender, generation, and the availability of DNA and brain scans.

Statistical Analyses: Analyses will be conducted using Statistical Analysis Software (SAS 9.1.3) (Carey NC). Primary outcomes will be (1) behavioral trait measures of impulsivity and aggression, and (2) DSM diagnoses of drug use disorder (including nicotine), alcohol use disorder, and disruptive behavioral disorders (Conduct Disorder and Attention Deficit Hyperactivity Disorder). These outcomes will be modeled as continuous and categorical variables respectively. I will however also explore alternative classifications, including the number of symptoms and age of onset for diagnoses, and number of cigarettes smoked per day. Continuous outcomes will be modeled using multivariate linear regression (PROC REG), and categorical outcomes using COX proportional hazards regression (PROC PHREG); both will account for length of follow-up, gender, and familial risk for depression as detailed below ("other variables and their rationale for inclusion or exclusion"). Prior to formal statistical modeling, I will plot the data to examine the distributions of variables. For variables not normally distributed, I will employ transformations to approximate normality or use non-parametric methods. For symptom counts, I will determine whether linear or Poisson models are appropriate.

		DNA N=306[1,2]	**MRI** N=214[1,3]	**DNA +MRI** N=173[1]
All	EXPOSED = 82	48	35	30
	UNEXPOSED = 264	118	109	75
	Total: 346	**166**	**144**	**105**
By Gender				
Male	EXPOSED = 45	28	20	17
	UNEXPOSED = 118	50	47	32
Female	EXPOSED = 37	20	15	13
	UNEXPOSED = 146	68	62	43
	Total: 346	**166**	**144**	**105**
By Generation				
Second	EXPOSED = 48	34	24	21
	UNEXPOSED = 118	61	52	39
Third	EXPOSED = 34	14	11	9
	UNEXPOSED = 146	57	57	36
	Total: 346	**166**	**144**	**105**

Table 5. Sample Size, stratified by smoking exposure, sex, generation, and availability of DNA, MRI scans, or both.

[1] Includes full sample regardless of availability of smoking data.
[2] DNA collection is still underway at the time of this submission and we anticipate obtaining DNA on up to 30 additional subjects. These will be included in the analysis, but are not reflected in the Ns above.
[3] Includes usable scans only.

The following section details the analyses and power calculations for each *a priori* hypothesis. The testing model for each hypothesis is also illustrated in **Figure 7**. Sample sizes used in the power calculations have been adjusted to account for the unequal distribution of exposed and unexposed offspring[160]. Power is calculated for continuous outcomes, under a false positive rate (α) of .05.

Hypothesis 1: Prenatal smoking will serve as the independent variable, and the aforementioned diagnostic and behavioral measures the dependent variables, while adjusting for age, gender, and familial risk for depression. The regression coefficients (for continuous outcomes) or hazards ratios (categorical) will provide a measure of the risk associated with prenatal smoking. With an adjusted sample of 250 (based on a 30% exposure rate in a sample of 346) and α=.05, I will have power of .98 to detect a moderate mean difference (cohen *d* = .5) in the sample, which is consistent with other effects sizes reported in the literature. Hypothesis 1B will be similarly tested, except that the dataset will be restricted to those with the disorder of interest, age at onset of the disorder will serve as the dependent variable, and prenatal exposure as the independent variable. With 50 subjects, I will have a power of .8 to detect a moderate difference in onset ages. I will also use a survival-based analysis to plot the onset trajectories for each group.

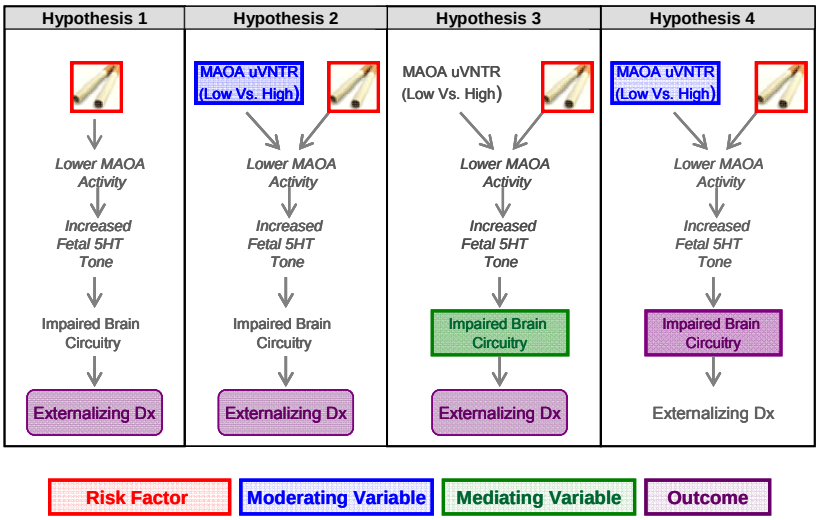

Figure 7: Illustration of the analytical framework for each hypothesis.

As an exploratory analysis, I will model the extent to which childhood behavioral measures are predictive of substance use disorders in adulthood. This will allow me to evaluate the roles of trait impulsivity and aggression as antecedents versus behavioral outcomes of substance use[161]. Furthermore, because the adult behavioral instruments (BIS-11, ZKPQ-III-R, and AQ) measure overlapping constructs and are likely correlated, I will also explore principal components analysis (PCA) methods to generate factors from the existing variables that most parsimoniously explain the greatest variance, and then use these new variables to test the original hypotheses.

Hypothesis 2: I will first examine whether MAOA genotype is related to diagnostic outcomes regardless of exposure (2A). The analysis is similar to Hypothesis 1 except that genotype rather than exposure will be the independent variable. To test whether the relationship between maternal smoking and offspring outcomes vary by genotype (2B), I will include genotype, exposure and a further genotype by exposure interaction term as independent variables in the model. Evidence of a formal difference will be provided by a significant interaction term. After adjusting for unequal proportions, I will have power of .83 at α=.05 to detect a moderately sized interaction (interaction F = .25).

Hypothesis 3: I will first compare the behavioral performance of exposed and non-exposed offspring on the task (3A). Reaction times will be entered as the dependent variable in an ANOVA, with stimulus (congruent/incongruent) the within-subjects factor and prenatal smoking the between-subjects factor. The hypothesized difference in performance between exposed and unexposed offspring will be tested by assessing the statistical significance of the diagnosis by stimulus interaction. To determine if prenatal smoking is associated with ROI cortical changes in response to the behavioral inhibition task (3B), a contrast mask (exposed vs. unexposed) will be incorporated in the SPM General Linear Model in which the incongruent minus congruent activity is the dependent variable. Analogous masks can be generated for each outcome variable to test their independent associations with the brain measures. Finally, to test for mediating effects (3C), the average ROI regression coefficients (or for functional connectivity analyses, the coherence coefficients), will be entered into a model testing the association between exposure and behavioral outcomes. The proportional decrease in ß of the exposure variable following inclusion of the imaging coefficients will be interpreted as the proportion of the association between prenatal smoking and offspring outcomes that is mediated by differences in brain circuitry related to the behavioral inhibition task. I will have a power of .85 to detect a moderate sized mediation[162] based on a Sobel Test[163]. If there is no evidence for mediation, I will examine the hierarchical contributions of exposure and brain circuitry on the diagnostic and behavioral outcomes.

Finally, I will perform an additional analysis using only those subjects who did not have any substance use disorders (SUD) at the time of the brain scan. Using this truncated sample, I will examine whether the functional brain differences identified in 3B predict subsequent onset of a SUD. This longitudinal analysis will strengthen the interpretation of my results by allowing me to distinguish brain changes that may predispose to

substance use disorders from those that may be a consequence of the disorder. There are currently 149 subjects with an MRI who have no SUDs. Assuming a 75% follow-up rate, I will have a power of .94 to detect a 20%, .83 to detect a 15% and .70 to detect a 10% incidence of a diagnosis in the 113 estimated subjects.

Hypothesis 4 is meant to be hypothesis generating rather than testing. For Hypotheses 4A and B, moderator analyses as described for Hypotheses 2 will be employed. Finally, clinical and behavioral outcomes will be modeled descriptively, stratified by prenatal exposure, genotype, and cortical signal changes. The independent variables will then be tested using stepwise regression models to determine the individual and combined contribution of each variable on the pathway to risk.

Alternative Hypotheses. Should I be unable to reject the null hypothesis for tests of moderation (Hypothesis #2) or mediation (#3), the component terms of each hypothesis (that is, individual associations of exposures, genotypes and brain activation measures with the diagnostic and behavioral outcomes, or with each other) will still provide valuable information. [There will also be greater statistical power to identify these differences, as the sample will need not be restricted to the individuals who have data on all the variables required for the final-level analysis]. I will also, regardless of the outcome of my primary hypotheses, use an analogous analytic framework to examine other genetic variants related to the nicotinergic (including the cholinergic nicotinic receptor subunit (*CHRN*) genes[30]) and dopaminergic (dopamine receptors, transporter and catechol-*O*-Methyl Transferase[26, 31-33]) families (or other genetic variants that I identify through the course of this Award period), which may be associated with partially or completely distinct neural circuits and behavioral outcomes. And finally, though the primary hypotheses are based on *a priori* selections of ROIs, should I be unable to detect significant differences within these ROIs, I will turn to a data-driven approach, examining the whole brain to identify regional clusters activated by the behavioral inhibition task, and then use those regions to further examine associations with exposures, genes and behavioral outcomes.

Other Variables and Rationale for their Inclusion or Exclusion: (i) Age: Offspring of smoking and non-smoking mothers did not vary significantly vary by follow-up length (26.9 vs 25.7 yrs; p = .53). However, to account for the possibility that follow-up length could be differentially associated with outcomes of interest, I will use a Cox Proportional Hazards (PH) model, which models the uncertainty resulting from differential follow-up. PH models can only be applied to categorical variables; for continuous measures, length of follow-up will be included as a covariate in the model. (ii) Gender: All analyses will adjust for gender. I will also examine each gender separately, as externalizing disorders are more prevalent among males[164] and may be etiologically different across the sexes (e.g., differential interaction with sex hormones[165]). For the MAOA, located on the x chromosome, females can be homozygous or heterozygous, but males are always hemizygous. I will combine female heterozygotes and high functioning homozygotes into a single category if contrast tests warrant so. (iii) Family Relatedness: Because analyses include more than one subject from the same family or extended family, potential correlation of outcomes between family members will be accounted for by using link models that adjust for clustered data[166]. The regressions will incorporate two links, one based on the nuclear family, the other the extended (common grandparents) For genetic analyses, I will also test outcomes using one randomly selected person per family. (iv) Other: I will examine the association between the mother's genotypes and her smoking during pregnancy, to rule out effects of passive gene-environmental correlations[82] (i.e., where maternal genotype is commonly driving her own propensity to smoke, and the offspring's behavioral problems, regardless of prenatal exposure) [*Caveat:* This will be on a partial sample, as only ~60% of the genotyped offspring have a mother who was also genotyped]. I will not adjust for maternal or paternal depression or substance use, or maternal smoking outside of pregnancy, as we have previously documented that these were unrelated to offspring outcomes, and did not confound the association between maternal smoking and offspring psychopathology. I will also not model maternal or offspring antisocial disorders due to extremely low prevalence in the sample (N = 3), or effects of population stratification, as the sample is entirely of non-Hispanic Caucasian (primarily Italian) descent.

D6. Strengths and Weaknesses. There are a number of strengths to this study, including the longitudinal and prospective design, the extensive battery of clinical, behavioral, family, and environmental variables, the blindness of the assessments, and the availability of biomarkers to test underlying mechanisms. There are also limitations, and while these do not diminish the essence of the study, the results will need to be interpreted in their light. First, smoking was assessed retrospectively via self-report. It is possible that some smoking mothers may have under-reported use; however this would have *decreased* the observed effect size. Alternatively, smoking mothers could have been more prone to report offspring problems; though unlikely, we cannot rule

this out. An independent biological verification (e.g., cotinine levels) would have been useful. However, studies have reported high correlations between cotinine and self-reported smoking among both pregnant and non-pregnant women, in both adults and adolescents, and across different cultures[167-170]. When there have been inconsistencies, these have erred toward under-reporting by smoking mothers[171]. Furthermore, cotinine measurements can themselves be vulnerable to timing of measurement[172]. Second, because the parent study is based on a high-risk design, and is not a community sample, findings may not generalize to the larger population. It is also an observational study, and findings cannot be interpreted as causal, as other unmeasured variables that could account for the relationships observed. And finally, given the multiple outcomes being tested, results may not all meet stringent thresholds for significance for independent hypotheses. Because this is a training grant, testing different layers of outcomes is important both for acquisition of pilot data and to further my expertise.

D7. Conclusions and Future Directions. This study employs imaging and genetic approaches to target the mechanisms of a well-documented epidemiological finding. By identifying how individual variation in exposure, genetic architecture, and brain circuitry predispose to behavioral and substance use problems, we may begin to identify those at greatest risks for these adverse outcomes, and thereby require the earliest intervention. This study can in the long term thus have both scientific and public health implications.

Of course, this proposal is only one approach to address what are complex behavioral phenotypes[173]. No single factor is expected to play an overwhelming role, and the many layers of interactions between multiple genes, exposures, and brain circuits that likely drive these disorders will be the purview of subsequent applications. Through the execution of the this research proposal and the training plan, I will develop a deeper understanding of the biological and behavioral substrates of substance use disorders, and use this expertise to design more targeted studies to tap into these substrates. Though maternal smoking served as the primary exposure vehicle of this proposal, I will examine other alcohol, drugs, and medication (e.g. SSRIs and MAOIs, which may share similar mechanisms related to serotonin perturbation) exposures. Exposures need not be biological either: early childhood exposures (e.g., parenting, adversity, maltreatment) can also affect long-term sbehaviors and substance use outcomes in ways that are genetically malleable, as Caspi et al showed with early life stress and the serotonin transporter gene[25]. With the additional training, I will be able to refine the classifications by different *diagnostic* (e.g., abuse versus dependence), *pharmacological* (drug vs. alcohol, different classes of prescription and illicit drugs) and *behavioral* (e.g., impulsivity, compulsivity, expectation, delay avoidance) criteria, and thereby more specifically target underlying biological processes. I will also use longitudinal study designs to follow prodromal or high-risk individuals in order to identify factors associated with the transition from casual use to addiction[110]. Finally, following my earlier experience studying factors affecting remission from mental disorders[14], I am very interested in addressing questions in the *clinical* domain (e.g., factors affecting onset, disease severity, treatment response, relapse).

To pursue these avenues, I will employ several concurrent strategies. First, I will have the option to build further waves atop the present study sample to test new hypotheses that are generated from this proposal. Other applications will require *de novo* subject recruitment and experimental paradigms. Still others will require clinical populations, to which I will be directed and guided by my mentoring team, particularly Dr. Levin. And finally, some questions may be best addressed in very large cohort studies (such as national healthcare and epidemiological datasets) that can allow examination of outcomes in population based samples with sufficient power and the ability to account for numerous biases. To this purpose, I will, under the guidance of my co-mentors, identify and begin to chart access to such data sets, focusing on those with existing, or easily incorporable genetic and/or imaging measures. One such example is the Finnish Birth Cohort detailed in my training plan, which has a wealth of maternal prenatal exposures and biomarkers, and detailed offspring records. Another is an NIMH sample of ~2900 subjects that I have previously worked with[10], which was collected to serve as a control population for a schizophrenia study, but in which a number of subjects met criteria for drug dependence. I believe that this multi-approach to targeting study samples will provide the greatest potential to address the questions at both an epidemiological and biological level. The training that will be afforded by this Award will help me to achieve these collective goals, develop the desired expertise, and complete my transition into a fully independent investigator.

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