INVESTIGATING THE PLEIOTROPIC EFFECTS OF THE APOLIPOPROTEIN VARIANT EPSILON 4 ALLELE ON THE SCHIZOPHRENIA SYNDROME

by

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Abstract

Schizophrenia (SCZ) is a severe and complex disorder that presents in young adults and evolves to chronicity causing pervasive deterioration in personal, social, and professional functioning. SCZ is better conceptualized as a syndrome as the concrete underlying etiopathological mechanisms have not been identified. Nonetheless, there are reports of subtle abnormal post-mortem findings, moderate brain deficits, and severe neurocognitive impairments.

Although the etiology of SCZ is unknown, evidence suggests a strong genetic contribution to the disorder. The apolipoprotein E gene (*APOE*) codifies for the apoE protein, which is involved in a wide range of functions from cholesterol metabolism to synaptic plasticity. One of the three main allele polymorphisms of *APOE*, *APOE*-ε4, is associated with increased risk of Alzheimer's disease, decreased hippocampal volume, and neuropathological findings. However, data in youth indicates that it may also be associated with cognitive performance or brain development.

Samples of post-mortem brain tissue from SCZ patients and healthy controls from Brodmann area 9 were used to quantify the amount of apoE, cholesterol, methylation of the promoter region of reelin, reelin mRNA, apoE receptor 2 (apoER2), and very-low density lipoprotein receptor (VLDLR). In addition, a sample of first-episode psychosis patients and healthy controls was recruited. Subjects underwent neurocognitive testing as well as brain imaging at baseline and 9 to 12 months after. Our data demonstrated higher levels of methylation of the reelin promoter region and decreased expression of apoER2 in SCZ samples, but there were no differences in apoE, cholesterol, reelin or VLDLR. In SCZ data suggested dysregulation of apoE and cholesterol in both grey and white matter. In FEP, there was severe impairment in

verbal memory, but *APOE*-ε4 was associated with improved verbal memory over time in SCZ. *APOE*-ε4 status, but not memory capacity was associated with smaller hippocampal volume. *APOE*-ε4 is associated with different phenotypes of opposing effects in SCZ and may be associated with the syndromic expression of the disorder.

Lay summary

Schizophrenia (SCZ) is a devastating illness that presents in young adults causing severe deterioration in personal, social, and professional functioning. Evidence suggests a strong genetic contribution to the disorder. The apolipoprotein E gene (*APOE*) is a gene involved in cholesterol metabolism and synaptic plasticity, and one of its alleles polymorphisms is associated with increased risk of Alzheimer's disease, decreased hippocampal volume, and neuropathological findings.

Brain tissue was used to investigate *APOE* and other molecules effect in SCZ. Also, first-episode psychosis patients were studied for presence of brain abnormalities and cognitive deficits over time. Our data demonstrated abnormalities in molecules in SCZ that are associated with synaptic plasticity, brain abnormalities in the hippocampus, and cognitive deficits in SCZ. One of the *APOE* polymorphisms was associated with cognitive improvement over time. *APOE* is a gene that may be associated with brain abnormalities as well as modify the clinical presentation in SCZ.

Preface

A version of chapter 2 has been published.

Vila-Rodriguez F, Honer WG, Innis SM, Wellington CL, Beasley CL. ApoE and cholesterol in schizophrenia and bipolar disorder: comparison of grey and white matter and relation with *APOE* genotype. J Psychiatry Neurosci. 2011 Jan;36(1):47-55. doi: 10.1503/jpn.090116. PubMed PMID: 20964956; PubMed Central PMCID: PMC3004975. I was responsible for the assay development and optimization as well as conducting all the experiments using ELISA, Western blot, and genotyping. I conducted all the statistical analyses and I wrote the manuscript, which was revised by co-authors for further input.

In addition, work reported in chapter 2 has been a part of a second paper that has been published. Clare L. Beasley, William G Honer, Alfredo Ramos-Miguel, **Fidel Vila-Rodriguez**, Alasdair M Barr. Prefrontal Fatty Acid Composition in Schizophrenia and Bipolar Disorder: Association with Reelin Expression. Schizophr Res. 2017 Jun 2. pii: S0920-9964(17)30309-2. doi:10.1016/j.schres.2017.05.033. PubMed PMID: 28583708. I was responsible for the apoE assay development and optimization as well as conducting all the experiments using ELISA, Western blot, and genotyping of *APOE*. I contributed to write the manuscript, its revision, and submission.

The Clinical Research Ethics Board at The University of British Columbia approved this research. Certificate number H06-70401.

A version of chapter 3 has been submitted for consideration.

Vila-Rodriguez, F; Lang, DJ; Su,W; Kopala, LC; Baitz, H; Gicas, K; Thorton, AE; Torres, IJ; Smith, GN; Barr, AM; McEwan, GW; Müller, DJ; Kennedy, JL; Honer, WG. Decreased hippocampal volume in first episode psychosis *APOE*-ε4 carriers.

I was responsible for the formulation of the research question, all statistical analyses. I wrote the manuscript, which was revised by co-authors for further input.

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A version of chapter 4 has been published.

Vila-Rodriguez F, Lang DJ, Baitz H, Gicas K, Thorton AE, Ehmann TS, Smith GN, Barr AM, Torres IJ, Kopala LC, MacEwan GW, Müller DJ, Kennedy JL, Honer WG. Verbal memory improvement in first-episode psychosis *APOE*-ε4 carriers: a pleiotropic effect? Neuropsychiatr Dis Treat. 2017 Dec 8; Vol 13: 249-257. PubMed PMID: 29263671; PubMed Central PMCID: PMC5727104. I was responsible for the formulation of the research question, all statistical analyses. I wrote the manuscript, which was revised by co-authors for further input.

The Clinical Research Ethics Board at The University of British Columbia approved this research. Certificate number H01-70454.

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List of Abbreviations

Aβ	Amyloid Beta
ADHD	attention-deficit hyperactivity disorder
AMPA	alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR	AMPA receptor
ACC	anterior cingulate cortex
AP	Antipsychotics
APOE	Apolipoprotein E, gene
apoE	Apolipoprotein E, protein
BA	Brodmann Area
BSA	bovine serum albumin
Chr	chromosome
CNS	Central Nervous System
CNV	copy number variation
COMT	catechol *O*-methyl transferase
CPZ	Chlorpromazine
CTRL	control
CtThck	cortical thickness
CVLT	California Verbal Memory Test
Da	Dalton
DA	dopamine

DLPFC	dorsolateral prefrontal cortex
DSM	diagnostic and statistical manual of mental disorders
DTI	diffusion tensor imaging
EEG	electroencephalography
EF	Executive Function
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FEP	first-episode psychosis
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
Hipp	hippocampus
IgG	immunoglobulin G
IHC	immunohistochemistry
IQ	Intelligence Quotient
kDa	kilo Dalton
L-DOPA	L-3 ,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MDD	major depressive disorder
mGluR	metabotropic glutamate receptor
MRI	magnetic resonance imaging
n.s.	non-statistically significant
NAA	N-acetyl-aspartate
NE	norepinephrine (noradrenaline)

NMDA	N-methyl D-aspartate
NMDAR	NMDA receptor
NSO	non-secreting mouse myeloma cell line (antibody supernatant control)
OFC	orbitofrontal cortex
PBS	phosphate-buffered saline
PC12	pheochromocytoma cell line
PET	positron emission tomography
PFC	prefrontal cortex
PMI	post mortem interval
PNOS	psychosis not otherwise specified
PNS	peripheral nervous system
PVDF	polyvinylidene fluoride
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCZ	schizophrenia
SD	standard deviation
SOS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNP	Single nucleotide polymorphism
TBS	tris-buffered saline
TBST	tris-buffered saline containing 0.05% v/v Tween-20
Tx100	tritonX-100
VM	Verbal Memory

v/v	volume by volume
w/v	weight by volume
WM	Working Memory

Glossary

Applied Science: Discipline of science concerned with the application of knowledge to practical matters.

Clinical Phenotype (Medicine): the set of signs and symptoms of a disease resulting from expression of a pathological process.

Complex system: A system consisting of multiple interconnected and interwoven parts.

Consolidation: Is the processes of stabilizing a memory trace after the initial acquisition. Usually two specific processes, synaptic consolidation (which occurs within the first few hours after learning or encoding) and system consolidation (where hippocampus-dependent memories become independent of the hippocampus over a period of weeks to years).

Crystalized intelligence: Is a principal manifestation of the influence of experience, education, and acculturation (also conceptualized as Verbal abilities/intelligence)[1]

Encoding: In the context of memory and learning, encoding is the initial process by which information is manipulated to facilitate learning.

Epistemology: The theory of knowledge, especially with regard to its methods, validity, and scope.

Fluid intelligence: Is an obvious and pervasive intellectual ability, heavily influenced by biological factors on intellectual development (e.g. heredity, injury to CNS, brain development) or to basic sensory structures (also conceptualized as Non-verbal abilities/intelligence) [1]

Memory Processes: Encoding, Consolidation, Storage, Retrieval/Recall

Nomenclature: the devising or choosing of names for things, especially in a science or other discipline; the body or system of names in a particular field.

Nosology: The branch of medical science dealing with the classification of diseases.

Phenotype (Biology): the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.

Pleiotropy (Gen): The production by a single gene of two or more different apparently unrelated phenotypes.

Pleiotropy, *Anagonistic* (Gen): The production by a single gene of opposing [beneficial and detrimental] phenotypes.

Proactive interference: is the detrimental effect of prior learning on the retention of subsequently learned material [2].

Pure Science: A Science depending on deductions from demonstrated truths or without regard to practical applications.

'No hold' test: Neuropsychological test sensitive to brain damage[3]

'Hold' test: Hold tests typically measure crystalized intelligence, that is stored knowledge and skills, such as vocabulary and pronunciation [3].

Retrieval/Recall: Recall or retrieval of memory refers to the subsequent re-accessing of events or information from the past, which have been previously encoded and stored in the brain. In common parlance, it is known as remembering. During recall, the brain "replays" a pattern of neural activity that was originally generated in response to a particular event, echoing the brain's perception of the real event.

Retroactive Interference: is the detrimental effect of learning new information on previously learned material[2].

Serial positioning effects, Primacy: In the context of memory research, this phenomenon by which material presented first is better recalled.

Serial positioning effects, Recency: Phenomenon by which the latest information presented is better recalled.

Serial positioning effects, serial clustering: Phenomenon where items are recalled in clusters (in California Verbal Learning Test [CVLT] whenever two words are recalled in the same order.

Set: Ability to respond adaptively and appropriately to a stimulus situation.

Storage: Storage is the more or less passive process of retaining information in the brain, whether in the sensory memory, the short-term memory or the more permanent long-term memory.

Taxonomy: the branch of science concerned with classification, especially of organisms; a scheme of classification.

Acknowledgements

I offer my deepest gratitude and respect to my mentor Dr. William G. Honer for his generous and patient guidance and advice along this long journey that started a long time ago. I have benefited from his insightful reflections and scientific guidance as much as I have admired his personal and professional ethos. I owe particular thanks to my supervisory committee members Dr. Alasdair M. Barr and Dr. Ivan Torres for their support and time devoted to this thesis. Special and sincere gratitude is owed to Dr. Donna J. Lang who has always helped with infinite patience and provided invaluable advice, guidance and support from the very beginning, and continues to do so.

I owe a public apology to my daughters Carla and Nora as well as my spouse Veronica for the absences they have patiently endured; Carla, Nora, and Veronica: I missed you every second I was away, but the memory of your smiles and hugs while away accompanied me and provided me with the strength of character I needed to accomplish this.

My parents have been a role model and an infinite source of love and support throughout my entire life. It might be obvious to say that I would not have accomplished anything in life without them, but it is not a trivial statement to make.

Dedication

To my parents, Ana and Jesus.

To my grandparents, Manuela and Corentino.

Chapter 1: Introduction

1.1 Nosology in psychiatry: still in the syndrome era

"*If you don't know history, then you don't know anything. You are a leaf that doesn't know it is part of a tree*" Michael Crichton.

Any scientific work meriting consideration should first define a clear epistemological frame of reference, including its methods, validity, and scope; therefore, this dissertation shall set its course by providing some fundamental definitions that serve as a frame of reference.

Applied sciences concern themselves with the application of knowledge to an action, whereas pure sciences serve the purpose to know[4]. Mathematics, Chemistry, or Physics are prime examples of pure sciences and rely on deductions from demonstrated truth(s) as their foundation. On the other hand, scientific disciplines such as Engineering, Economics, or Medicine draw from diverse pools of knowledge, including the pure sciences, to find solutions to the challenges they face. Medicine as a science is primarily concerned with human disease: identifying, classifying, treating, or preventing disease are some of the main objectives of Medicine as a field in the application of knowledge into action.

Disease is a medical term used to denote a discrete and unique pathological process or state. The foundation of the concept of disease in its simplest terms can be formulated as:

Disease: Unique and Known Etiology → Set of Signs and Symptoms

The external manifestations of a disease are composed of signs and symptoms; we could also borrow the concept *phenotype* from Biology, and use the term *disease phenotype*. Whereas a sign is an objective manifestation of the disease that can be positively observed, such as an increase in body temperature, or disorganized or catatonic behavior in psychosis, a symptom is a subjective account of an inner experience, such as pain or sad mood, related by the person. Diseases tend to

present in a prototypical manner where a particular constellation of signs and symptoms present together in a regular pattern called a syndrome. A syndrome is a prototypical and patterned presentation of sign(s) and/or symptom(s) that can be recognized. The recognition of syndromes is fundamental in the process leading to diagnostic formulation or diagnostic hypothesis, which in turn guides the formulation of a treatment plan. A diagnostic formulation may be tentative /provisional and treatment might proceed without a definite and certain etiology having been demonstrated. An example of this approach is the common cold where a patient presents with a set of sign(s) and/or symptom(s) (e.g. cough, malaise, fever), the presumptive diagnosis is made and treatment recommended (e.g. analgesics for pain and fever, rest); the specific etiology is not demonstrated but rather presumed (e.g. in the case of common cold the most likely etiology is viral). In many instances, however, the specific etiologic agent/cause is pursued with supplementary tests that provide additional information (e.g. diagnostic tests). Diagnostic tests provide objective information on features that aid in diagnosis (i.e. are biomarkers). The information obtained with diagnostic tests could be considered a particular type of sign. In lung cancer, a patient might present with a constellation of sign(s) and/or symptom(s) (e.g. productive cough with blood on sputum, malaise, fever, weight loss, fatigue). This syndrome may lead to a presumptive diagnosis of a lung malignancy, but no treatment is initiated until further evidence is gathered to specifically demonstrate the etiology. In this example, an imaging test may be ordered and demonstrate a mass in the right lung. Subsequently, a biopsy of the mass can provide histological evidence that the mass is in fact a small cell carcinoma of the lung, and at this point a specific treatment plan can be formulated or further tests pursued to aid in formulation of a treatment plan (e.g. further imaging to stage the oncological process).

There are several factors that determine when, and how aggressively, the specific etiology of a disease is pursued, but perhaps two of the most relevant are i) severity of the illness, and ii) whether reliable biomarkers exist for a particular disease. Related to the availability of biomarkers is whether the etiology of the condition is known. Note that per definition of disease, the etiology must be known; consequently, medical conditions for which the specific etiology is not yet known are necessarily considered syndromes (i.e. a recognizable pattern of sign(s) and/or symptom(s) that present in a prototypical fashion).

Primary psychiatric conditions are the most notable example in medicine of ailments where the specific etiology is yet unknown and where reliable biomarkers are lacking.
In other words, diagnostic formulations up to the present day have been largely based on the recognition of a prototypical presentation of a constellation of sign(s) and/or symptom(s); therefore, the majority of primary psychiatric conditions are syndromes.

The Diagnostic and Statistical Manual of Mental Disorders, currently in its fifth edition (DSM-5), is the standard tool used for diagnostic purposes in North America, and its use has spread to many other parts of the world[5]. The DSM fully, explicitly, and openly acknowledges that psychiatric disorders are syndromes: "*A mental disorder is a syndrome characterized by clinically significant disturbance in an individual's cognition, emotional regulation, or behavior* ..." and further acknowledges that the categories described in the manual may not identify homogeneous groups of individuals who share a common and unique etiology[5]. The rationale for adopting this syndrome-based diagnostic approach is based on the practical utility of diagnostic criteria in meaningfully grouping individuals into categories that aid in diagnosis and treatment planning. In other words, the approach is directly related to the fact that psychiatry, as a discipline within Medicine, is an applied science and clinicians need to classify medical

conditions in a meaningful way even when facing conditions for which the etiology remains unknown.

It is beyond the scope of this introduction to provide a detailed and in-depth account of historical events that led to the current content and format of the DSM-5. However, a brief historical analysis will provide evidence in support of the thesis that the DSM is a practical tool to reliably operationalize the recognition and treatment of psychiatric conditions, but may be suboptimal when used with the objective to elucidate the etiology of psychiatric conditions.

The 19th century is a critical period in understanding Psychiatry as a discipline, as well as the method used to define psychiatric disorders, namely descriptive psychopathology[6]. The 19th century was a period in medicine when the anatomo-clinical conception of disease solidified, whereby lesions, clusters of signs/symptoms, and course of illness became the pivotal elements to define *disease*[7]. Bénédict Morel (1809-1873) and Karl Ludwig Khalbaum (1828-1899) are considered to be pioneers in implementing this model to psychiatry, and hence starting the shift from the non-medical, magical, or esoteric explanations for psychiatric conditions that existed prior to the 19th century (e.g. 'madness', 'insanity', or 'folia', to name a few) to conceptualizing the manifestations in these individuals as the result of an underlying [biological] pathological process. This shift resulted in the emergence of terms to describe and classify this new model of disease, and the adoption and creation of a *lexicon* to articulate the underlying concepts. This organic development of the *lexicon*, arising in parallel from diverse individuals and schools of thought without a common systematic frame, necessarily resulted in a disjointed array of terms that eventually complicated communication as well as recognition and treatment of psychiatric conditions. In the United States (US), this situation led the New York Academy of Sciences to initiate the development of a standard *nomenclature* in 1927 (of note, this initiative encompassed

all fields in Medicine, not just Psychiatry). In 1928, the first National Conference on Nomenclature occurred. Three years later, a trial edition of the proposed new nomenclature was tested in hospitals. In 1933, the first edition of the Standard Classified Nomenclature of Disease was published (in it there was a section on psychiatric conditions, "Diseases of the Psychobiologic Unit"). In parallel, and also during the first third of the 20th century, the American Psychiatric Association (formerly the American Medico-psychological Association) initiated efforts to gather uniform statistics of psychiatric conditions in hospitals, including the development of a classification primarily for statistical purposes. Based on this effort, the National Committee for Mental Hygiene (a US national organization in the beginning of the 20th century whose mandate was to promote mental hygiene) introduced a classification and statistical system in hospitals in 1927 called the "Statistical Manual for the Use of Hospitals for Mental Diseases". Eventually, the APA assumed responsibility for the publication of the Manual and in 1952 published the first edition and changed the title to "Diagnostic and Statistical Manual for Mental Disorders". The nomenclature of the DSM-I constituted the "Diseases of the Psychobiologic Unit" from the 4th edition of the aforementioned Standard Classified Nomenclature of Disease. Of note, the term 'disorder' was introduced in the DSM-I and distinguished from the term 'disease' used by its predecessors, by precisely delineating its conceptual meaning: "*This diagnostic scheme employs the term 'disorder' generically to designate a group of related psychiatric syndromes*" [8]. This historical context leading to the first edition of the Diagnostic and Statistical Manual is represented in Figure 1.1. In this Figure I provide further context to understanding the influence of two key players in the formulation of the concept of schizophrenia (SCZ) as a disorder in the DSM: Emil Kraepelin and Eugene Bleuler. I shall return to them in section 1.2.

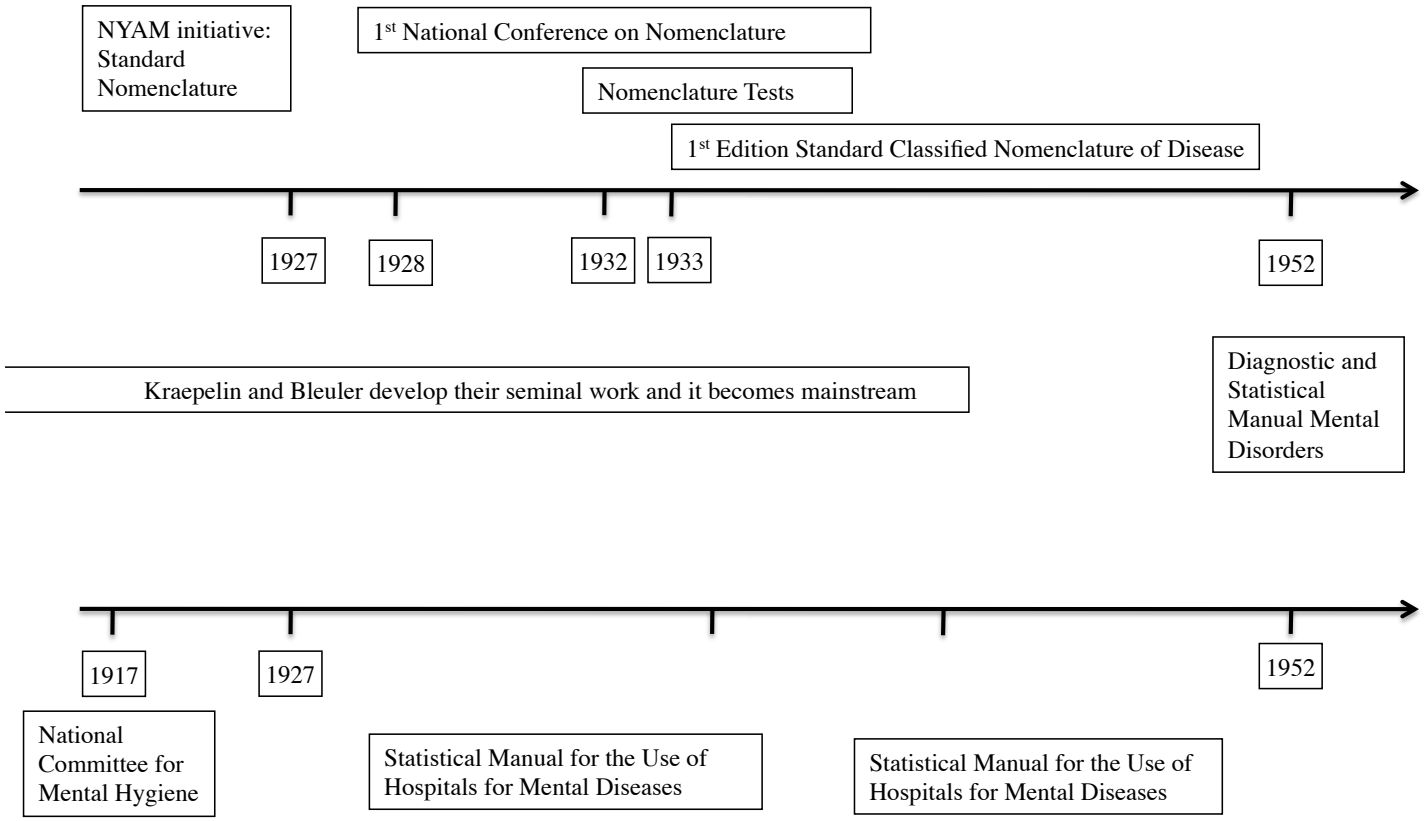

Figure 1.1 Nomenclature, taxonomic, and classification developments in psychiatry. This figure provides a historical timeline of events that lead to the creation of the diagnostic and statistical manual of mental disorders.

This brief historical account demonstrates that psychiatric conditions started to be acknowledged as *bona fide* medical conditions with an underlying, although not yet known, biological cause, which led to the need to create a nomenclature and a taxonomy (i.e. a nosology) that would allow clinicians to reliably identify these conditions and consistently guide decisions with regards to treatment. The elucidation of the biological mechanisms or causes underlying these psychiatric conditions was never the primary goal of the nosology tools; rather, it was hoped or assumed that adoption of a consistent nosology would be followed by the elucidation of an etiology, and change the status of psychiatric disorders to that of psychiatric diseases.

The fact that most primary psychiatric disorders are syndromes has profound implications both for clinical practice, as well as for any research effort aiming at investigating the etiopathological mechanisms operating in them. Figures 1.2 and 1.3 map out the concept of disease and disorder, and they illustrate how these concepts shape clinical practice, treatment, and research. Figure 1.3 shows how mental disorders (i.e. syndromes) can be helpful to classify sign(s) and/or symptom(s) and lead to a diagnosis that guides therapeutics without any necessary connection to biomarkers or investigation of etiological factors. This independent cycle, disconnected from the search for biomarkers or etiopathogenic mechanisms, can self-perpetuate as long as it yields a classification system that operationalizes diagnosis consistently and guides therapeutic strategies that are sufficiently effective. Incorporating biomarkers or investigating etiopathogenic mechanisms may be challenging when the starting point is syndrome clusters guided by the principles of operationalization. This disconnection is paramount to understanding the epistemological context in which this dissertation operates, since the object of study revolves around the potential etiopathogenic role of the apolipoprotein E varepsilon 4 allele in the SCZ

disorder (i.e. syndrome). By classifying our study participants based on descriptive psychopathology that facilitates clustering, we may be introducing a major source of noise that can potentially obscure discerning the role of such etiopathological factor.

However, this very fact is precisely why tackling such questions is critical to making progress in the field. Understanding what effect the apolipoprotein E varepsilon 4 may have on the clinical presentation, course, or treatment of the SCZ syndrome is a step to break the self-perpetuating cycle presented in Figure 1.3. If valid biomarkers are added the field will eventually transition from the conceptual framework of Disorder (Figure 1.3) to that of Disease (Figure 1.2).

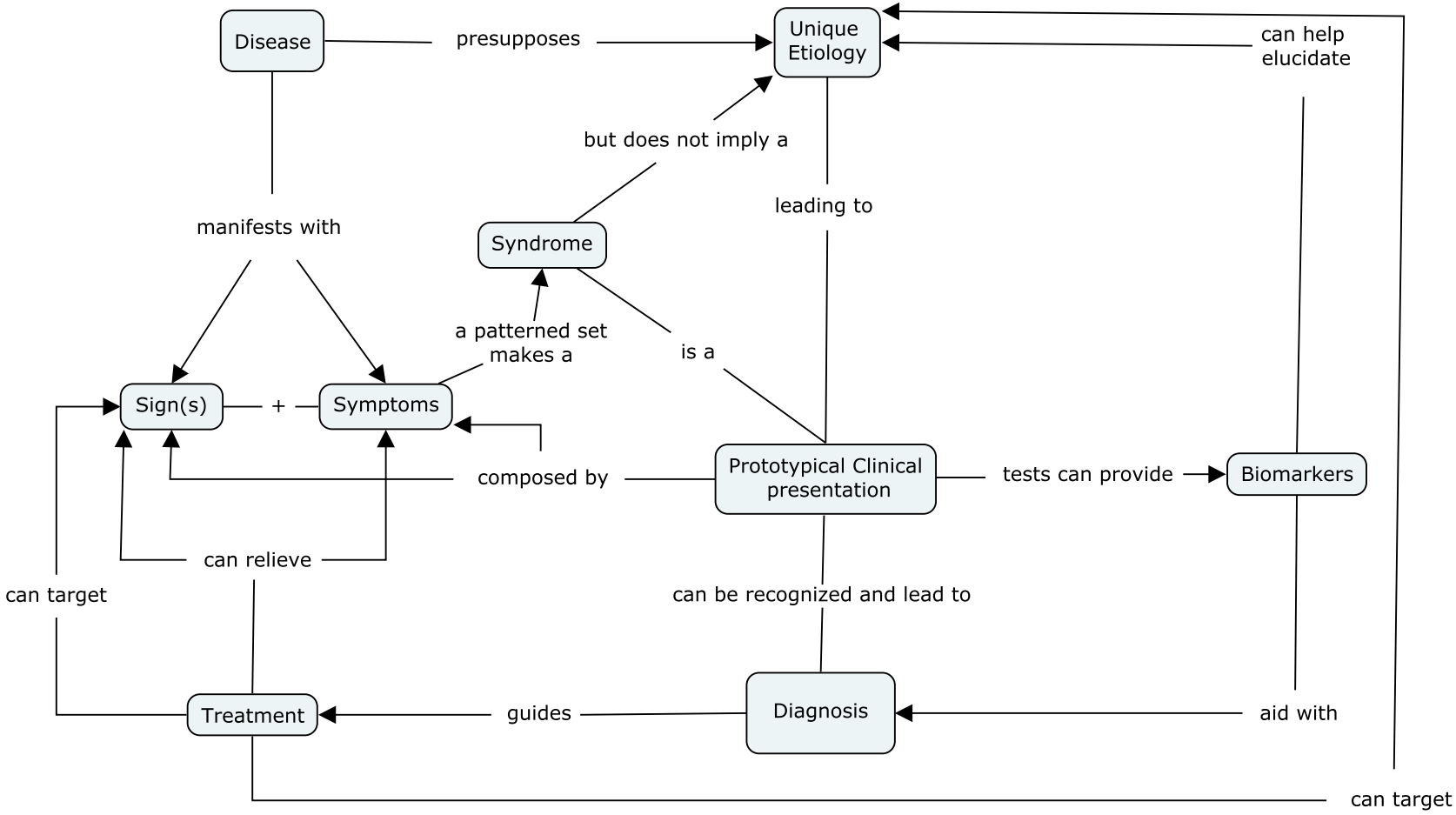

Figure 1.2 Disease concept map. The concepts that construct the idea of disease and the relationship of disease concept with other key related concepts are provided.

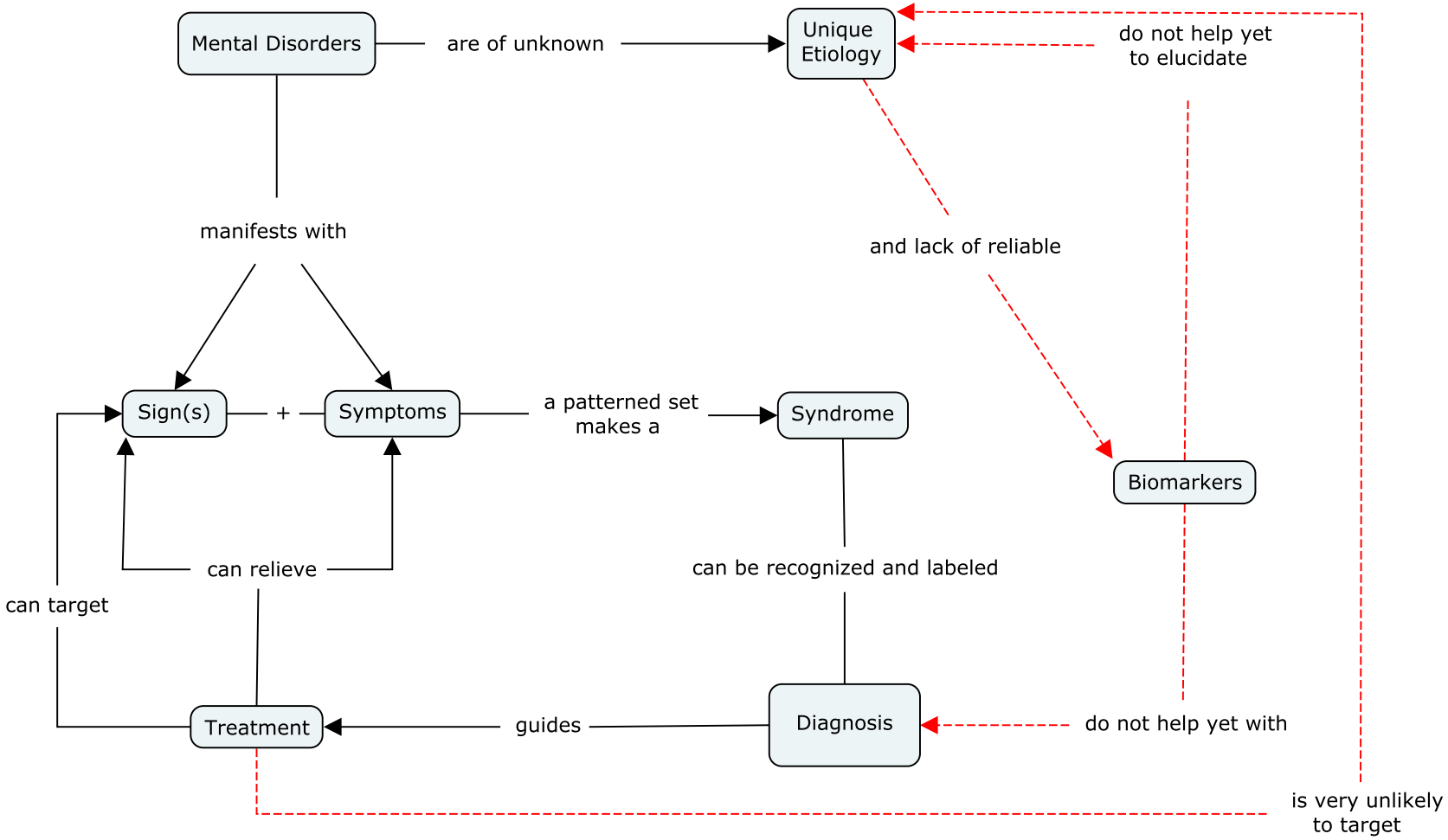

Figure 1.3 Mental disorder concept map. The concepts that construct the idea of disorder is provided and contrasted with the concept of disease by showing the different relationship of disorder concept with other key related concepts are provided.

1.2 The schizophrenia syndrome

In the previous section, a general framework to understanding the concept of mental disorders was provided. This framework will be essential in elaborating on the concepts of *psychosis* and *schizophrenia*. Clearly understanding how the concepts of *psychosis* and *schizophrenia* emerged and consolidated is of the essence not only to understand and interpret the experimental results of this dissertation, but also to interpret and understand current research in the field of schizophrenia, its limitations, and future directions for the field to progress[9]. The historical context of the concepts of *psychosis* and *schizophrenia* presented in this section is provided as a conceptual frame of reference for this dissertation. For a detailed and in-depth review of the history of the concepts of *psychosis* and *schizophrenia* I suggest, and have relied on, the works of German Berrios[6,7,10], Dominic Beer[11], Eugen Bleuler[12], Martin Bürgy[13], Emil Kraepelin[14], Bartolome Llopis[15], and Heinrich Neumann[16].

Prior to the 19th century, in most cultures across time, it was recognized that humans could suffer from severe and fluctuating disturbances expressed in abnormal and bizarre behavior(s) and these behaviors were equated to the idea of "madness" as well as other non-medical explanations (e.g. evil possession)[6]. In the 19th century, a conceptual shift occurred and those behaviors started to be seen through the lens of the medical model and as medical conditions, and terms such as "insanity" (which had legal connotations), "alienation" (originating in France) and "dementia" emerged. Constatt (1841) and Feuchterleben (1845) are credited with first introducing the term psychosis. In their descriptions, they both presumed a somatic etiology for psychosis or a "weakness of the brain"[17]. According to Berrios[7], the success of the term psychosis was facilitated by the change in the concept of disease at the time (i.e. to the anatomo-

clinical conception of disease), as well as by the need to develop a nomenclature and a taxonomy in psychiatry. While these factors set the need for a unifying term, they do not explain why this particular one gained rapid acceptance and prevailed over many others that were proposed at the time. I would hypothesize that an additional reason for its adoption is that the word "psychosis" has a direct translation and easy phonetic equivalency across the most influential western languages in use at the time, namely English (psychosis, /sīˈkōsəs/), French (psychose, /psikoz/), German (psychose, /psy'ço:zq/), Spanish (psicosis, /sikósis/), and Italian (sicosi, /si'kOzi/).

The formation of the concept of psychosis was also heavily influenced by the concept of delirium. Delirium had been a condition recognized for centuries as an acute state that presents with delusions, hallucinations, and disorganized behavior and to be directly associated with a concrete underlying medical condition. It is very plausible that clinicians used delirium as a template for the concept of psychosis[6]. Indeed, some forms of delirium were termed as "acute forms of insanity", and before the term psychosis gained acceptance, to describe psychosis some had used the concept of "delirium without fever". It is very important to note how the concept of psychosis (and later that of schizophrenia) became tightly bound to its syndromic presentation early on, and particularly to delusions, hallucinations, and disorganization. Once the concept crystalized, clinicians rapidly started to identify variations of the archetypical presentation of psychosis, and under the implicit or explicit assumption of different biological etiological factors, proposed the existence of discrete nosological entities (i.e. presumptive diseases). The list of these variants is extensive, but to name a few that are highly relevant to this work, I will note "cycloid psychosis" (Khalbaum), "manic-depressive psychosis" (Kraepelin), "dementia praecox" (Kraepelin), and "Schizophrenia" (Bleuler).

In contrast to this view of discrete nosological entities, there was a current of thought arguing for a unitary concept of psychosis[15,16]. This school of thought emphasized the commonalities amongst the various clinical presentations and argued that the diverse clinical presentation resulted from differences in the expression of the same fundamental underlying process. The argument was that at the core of the psychosis there is a disturbance of consciousness that affects the *content*, rather than the *state* of it (i.e. as in the neurological sense), and they posed that the resulting clinical presentation, delusions, hallucinations, etc., was secondary to this disturbance of consciousness and its ability to apprehend reality[15]. The Unitary Psychosis school of thought came closer to addressing the primary question of "*what **is** psychosis*?" Of note, current medical dictionaries, as well as electronic sources such as Google and Wikipedia, all point to the disconnection with reality as a core aspect of their definitions of psychosis[a]. In contrast, DSM-5 provides an operational, and tautological, definition of *psychosis* by stating "[Psychosis] *refers to delusions, any prominent hallucinations, disorganized speech, or disorganized or catatonic behavior* (…)" where hallucinations, disorganized speech or disorganized/catatonic behavior are all diagnostic criteria that operationalize the diagnosis of psychotic *disorders*[5]. In other words, no attempt is made to define, delineate, or clarify what the concept of *psychosis* itself is.

I have provided the historical landscape that led to the conceptualization of psychosis as a medical condition, as well as the contextual need for a nomenclature and taxonomy in psychiatry. The foundation of our current definition of the concept of schizophrenia can be

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[a] "Psychosis is a symptom or feature of mental illness typically characterized by radical changes in personality, impaired functioning, and a *distorted or nonexistent sense of objective reality*"

traced to the late part of the 19th century and the early part of the 20th century, which is when the concept crystallized. Emil Kraepelin (1856-1926) is one of the most influential figures to understand both modern psychiatry as well as the concept of schizophrenia. Kraepelin used the term "dementia praecox"[b] to designate a group of clinical presentations he presumed were caused by "internal causes" (i.e. a somatic/biological etiology, yet unknown), and that presented with severe deterioration in functioning. He argued general paralysis, 'senility, or epilepsy had a similar profound deterioration and thus the term 'dementia' was a good fit, and added the term "*praecox*" (premature[c]) to convey the fact that the condition presented in youth as opposed to the other forms of 'dementia'. In the introduction of his notable treatise '*Dementia Praecox and Paraphrenia*', he pointed to the core aspect of the condition as a "*peculiar destruction of the internal connections of the psychic personality*" which manifest "[predominantly] *in the emotional and volitional spheres of mental life*"[14]. Kraepelin, following the descriptive phenomenological approach, systematically and comprehensively detailed the *entire* clinical presentation of the condition. He provided a fully rounded description of both the features that were present and associated with impairment, as well as those that were absent. Kraepelin did not make a case for a strict hierarchy of signs/symptoms, nor did he provide any quantification of their frequency. His description of both hallucinations and delusions was among the most

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[b] The term 'dementia praecox' had been used before him by Morel (1860) and Pick (1891), a fact Kraepelin himself acknowledges in his work.

[c] Although the terms 'praecox' is often translated to English as 'early', it is more accurate to use the term 'premature', as the term was to signify the onset of this disease to occur during or shortly after puberty and therefore way before the regular onset of dementias.

extensively elaborated of all the symptoms, and he did confirm the fact that these were very frequent and were subsequently framed as the main symptomatic domain in his description. The other essential aspect of Kraepelin's definition had to do with the course and prognosis of the illness, and he made this a pivotal aspect to separate schizophrenia from manic-depressive psychosis. Whereas Dementia Praecox was argued to lead to full deterioration and consistently associated with poor prognosis, Manic-Depressive psychosis was associated with full recovery and better prognosis. This distinction proved to be very helpful as it allowed clinicians to make predictions in a large number of patients, thus supporting the validity of the concept. As I will discuss now, Bleuler qualified Kraepelin's strong stance with regards to the disease course and prognosis[d].

Eugen Bleuler (1857-1939), in his monograph "*Dementia Praecox oder die Gruppe der Schizophrenien*"[e], not only renamed the disease to its current term "schizophrenia", but more importantly, he contributed to further characterize and refine its nosological boundaries[12]. First, Bleuler implied in his description a hierarchy of symptoms, since he segregated fundamental vs. accessory symptoms. Fundamental symptoms were described as specific, always present, and stable over time, whereas accessory symptoms were considered secondary or a by-product of

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[d] Kraepelin himself in 1920 qualified his initial view as too rigid in light of the presence of intermediate forms of the disease [7].

[e] The first edition of this book was published in 1911, but it was not until 1950 that is was translated and published in English. Nonetheless, Bleuler published his "Textbook of Psychiatry" in 1916 in German, and the section on the "Group of Schizophrenias" was already there. This textbook was later translated to English in 1923 and published in North America in 1924.

fundamental symptoms. In other words, Bleuler argued for fundamental symptoms to be pathognomonic of schizophrenia without explicitly making that claim. With regards to the course of the disease, schizophrenia was defined as a condition where deterioration was expected, but took a subtle shift from the more absolute and quantitative view of Kraepelin where Dementia Praecox would always progress in full to 'dementia'. Bleuler's position was more qualitative as he introduced the notion that it was the **absence** of full recovery or complete return to baseline that characterized the disease (i.e. *restitutio ad integrum*). He noted the presence of residual symptomatology as a key aspect to the definition of schizophrenia. The third core aspect of his definition of the disease was the observation that the common denominator of all fundamental symptoms was the impairment in integrating mental functions, or in his own words, "a clear-cut splitting '*abgespalten'* of the psychic functions" (and hence his choice of the suffix 'schi').

Bleuler's fundamental symptoms included thought process ('Associations'), affect ('Affectivity'), impairments in will ('Ambitendency', 'Ambivalence'), and the relation to the external world ('Autism'). Heavily influenced by Freud's conceptualization with regards to mental activity, he provided detailed and exquisite descriptions of what he considered abnormal 'Associations' or what we currently refer to as thought process abnormalities such as thought blocking, loose associations, or non-sequitur thinking. His description of abnormalities in affect included three dimensions i) the deterioration of affect and progressive absence of it, ii) loss of ability to modulate affect, and iii) the disconnection between affect and content in the stream of consciousness, known as incongruent affect in contemporary terms. With regards to accessory symptoms, Bleuler extensively covered a wide array of psychopathological phenomena, but similar to Kraepelin, acknowledged that hallucinations and delusions were very frequent,

"almost present in all hospital patients" and that invariably these were the symptoms that lead to hospital admission.

In summary, both Kraepelin's and Bleuler's perspectives had several key aspects in common:

1. Both presumed an underlying neurobiological cause(s) not yet known,
2. These biological causes would lead to a common **group** of illnesses, even though they both used a singular term to describe the disease,
3. This group of illnesses had in common a deteriorating course and a severe impact on functioning,
4. Hallucinations and delusions are considered to present very frequently and are very amenable to be captured,
5. Disturbances in thought process, affect and volition are considered core manifestations,
6. The underlying mechanism leading to #5 is vaguely, but consistently defined by both: "*Peculiar destruction of the internal connections of the psychic personality."* [14] and "*clear-cut splitting of the psychic functions*." [12]

Now that I have described the historical context surrounding the efforts and need to develop a nosology in psychiatry, as well as the foundational work of Kraepelin and Bleuler, the connection between the two streams becomes obvious. The impact of that connection to our contemporary conceptualization of schizophrenia (and psychiatric disorders) is also transparent. Namely, Kraepelin and Bleuler provided a systematic and comprehensive characterization of the clinical manifestations of a group of diseases which facilitated the inclusion of this concept in the

nosological classifications of their day, as their descriptions proved to be useful and valid to clinically separate clinical conditions, creating the illusion of disease status; and yet, the lack of a defined and concrete etiology for these conditions made them syndromes *de facto*.

Another unforeseen consequence of this historical development is that hallucinations, delusions, and behavioral disorganization gained a prime status in the diagnostic process for pragmatic reasons in facilitating diagnosis, and not because they were considered closer to the primary etiological cause(s) of the disease(s). This situation has remained unchanged up to the very latest edition of the DSM (DSM-5), where the A. criteria ("*Characteristic symptoms*") for schizophrenia include: i) delusions, ii) hallucinations, iii) disorganized speech, iv) grossly disorganized or catatonic behavior, and v) negative symptoms[5].

1.3 Cognitive impairment in schizophrenia

Cognitive impairment is not a diagnostic criterion in DSM-5, and nor was it in its prior editions. Instead, in the DSM-5 cognitive deficits are discussed as an associated feature that supports the diagnosis. It is acknowledged that these deficits are frequent, impact functioning, and encompass several domains rather than a concrete or isolated cognitive ability[5]. The historical context provided in Section 1.1 on the development of nosological classifications and the development of the concept of psychosis/SCZ showed how positive symptoms (i.e. delusions, hallucinations) took precedence for pragmatic reasons to the detriment of other manifestations of the disorder. An additional factor that may have particularly influenced the secondary role assigned to cognitive deficits is that the epistemological boundaries of the field of

neuropsychology did not fully crystallize until the mid-1950s[f]. In addition, neuropsychology initially developed in the context of studying the impact of localized brain lesions on specific cognitive abilities. The subsequent integration of neuropsychological methods of investigation in SCZ (and other mental disorders) was a progressive addition over the second half of the 20th century. It became apparent that interventions introduced to treat SCZ, namely electroconvulsive therapy in 1938[18] and chlorpromazine in 1952[19,20] , mainly treated positive symptoms of the illness reinforced their pragmatic value to the detriment of other symptoms (e.g. cognitive deficits).

Currently, cognitive deficits are recognized as a core trait associated with decreased functioning in SCZ[21]. These deficits have large effect sizes, and a subset of them are comparable to those observed in patients with dementia[22]. Usually, deficits encompass a broad range of cognitive abilities including attention, verbal and non-verbal memory, executive function, working memory, processing speed, and psychomotor abilities[23]. Verbal memory, working memory, executive function and attention are amongst the most consistently and most impaired cognitive domains, although specific deficits occur in the context of a generalized deficit in the background. The type and effect size of cognitive deficits has been shown to be present and very consistent in first-episode SCZ.

The profile of cognitive deficits in SCZ has been proposed to serve as a potential endophenotype. Examples of proposed endophenotypes include the distinction of deficit vs. non-deficit SCZ[24], or the distinction between pervasive deterioration/ declined IQ/sparingly

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[f] Karl Lashley is credited as the father of neuropsychology due to his foundational work and use of the term [400]

affected[25], see Table 1.1. Deficit SCZ is defined as pervasive and enduring negative symptoms and pronounced cognitive impairment[26]. A recent meta-analysis looking at the neuropsychological deficit profile of SCZ based on this subdivision found that deficit SCZ was associated with more widespread cognitive deficits as well as more pronounced deficits in areas of social cognition, verbal fluency, and processing speed compared to non-deficit SCZ. Similarly, Weickert and colleagues described a subgroup of SCZ patients (pervasive deterioration) characterized by a generalized and severe cognitive deficits and low premorbid IQ (<90). In contrast, they also described a group of patients who presented with normal premorbid IQ, but experienced a decline of >10 points on IQ upon onset of psychotic symptoms with deficits predominantly in working memory, executive function and memory (declined IQ), and a third group who had normal premorbid and current IQ who presented with more selective and subtle deficits in working memory and executive function[25]. Of note, the putative dysfunctional brain areas underlying each of these proposed endophenotypes are hypothesized to be different and thus, may potentially have an etiopathogenic correlate (see Table 1.1).

	Premorbid IQ	Drop in IQ	Cognitive Domain	Anatomical substrate	Hypothesized Etiopathogenesis
Group 1 Deteriorated	Below	No	Wide-spread	Pan-Cortical	Major genetic abnormalities or in utero insults
Group 2 Drop IQ	N	Yes	EF, WM, Att Memory	Frontal Temporal	Minor genetic factors and epigenetic mechanisms
Group 3 Subtle deficit	N	No	EF/WM	Frontal	Autoimmune

Table 1.1 Description of potential endophenotypes based on type and severity of cognitive deficits and hypothesized underlying etiopathogenesis. Based on and modified from [25]. IQ: Intelligence Quotient; EF: Executive Functioning; WM: Working Memory; Att: Attention.

The identification of endophenotypes based on cognitive deficits is not the only valuable line of inquiry that may lead to a better understanding of the etiopathogenesis of SCZ. The timing of onset and the course of cognitive deficits may also provide critical clues. Cognitive deficits have been shown to be present long before the onset of psychotic symptoms, in the prodromal state, and the largest impact or deficit appears to occur before or around the first psychotic episode and subsequently remains fairly stable for one or two decades[27]. A further wave of cognitive deterioration occurs after the fifth decade of life, and in selected subgroups of patients, progresses to a severe deterioration[28]. The precedence of cognitive deficits before the onset of psychotic symptoms would support the hypothesis that underlying pathogenic mechanisms of cognitive impairment might play a primary role, and that they might secondarily lead to psychotic symptoms if or when additional mechanisms come into play. This hypothesis would be congruent with the diathesis-stress model of SCZ, whereby an underlying neurobiological factor (e.g. gene variation – diathesis) would require a "second hit" (i.e. stress) to trigger the development of the disorder[29]. This model accounts for, i) less severe cognitive deficits have been observed in non-psychotic relatives of patients with SCZ, and ii) these deficits have been shown to have a correlation with the degree of shared genetic load as indexed by blood closeness between two individuals[30–32].

The overlap between the degree of blood proximity and cognitive deficits in unaffected relatives clearly points to genetic mechanisms underlying cognitive deficits. Elucidating which genes may be associated with cognitive deficits is a fundamental step to elucidating the neurobiology of these deficits as well as the etiopathogenesis of SCZ.

1.4 Apolipoprotein E (apoE)

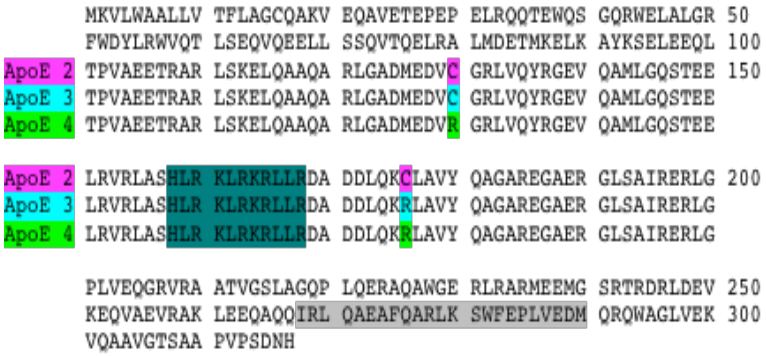
The apolipoprotein E gene (*APOE*) varepsilon 4 is one putative genetic variation that plausibly may play a role in the etiopathogenesis of cognitive deficits and brain abnormalities.

1.4.1 The apolipoprotein E gene *(APOE)*

Located in chromosome 19 in humans, the *APOE* has three main allele variations due to a single nucleotide polymorphism (SNP) present in germ line cells, namely *APOE*-ε2, *APOE*-ε3, and *APOE*-ε4[33–35]. The gene codifies a 317aa arginine-rich, two-domain protein that differs by a cysteine-arginine interchange in one of two positions at residues 130 and 176, leading to the three protein isoforms[33–36]. The protein undergoes intracellular cleavage (299aa), glycosylation with sialic acid, and secretion[37]. The tertiary structure of the protein, as well as relevant motifs and regions in the protein, is presented in Figure 1.4. There are 45 *APOE* variants identified (NCBI ClinVar database, accessed July 15, 2018, https://www.ncbi.nlm.nih.gov/clinvar?term=107741[MIM]). The majority of allelic variants are associated to familial forms of hyperlipoproteinemia type III (e.g. APOE, 1-BP DEL, 2919G or NM_000041.3(APOE):c.[487C>T;91G>A]), but other *APOE* variants have been associated to lipoprotein glomerulopathy (e.g. NM_000041.3(*APOE*):c.488G>C (p.Arg163Pro)). The allele frequency of the top three most frequent variants in the population is 60-70% (*APOE*-ε3), 15-20% (*APOE*-ε4), and 5-10% (*APOE*-ε2). There are allele frequency variations as a function of latitude and ethnicity with a negative correlation between frequency of *APOE*-ε4 and *APOE*-ε3 (i.e. the higher the frequency of *APOE*-ε3 the lower the frequency of *APOE*-ε4, and vice versa), and a higher proportion of *APOE*-ε4 in northern Europe compared to both the Mediterranean basin and indigenous populations in America, Oceania, and Africa. Of note, in Africa, populations closer to the Mediterranean basin have lower proportion of *APOE*-ε4 compared to

populations in central and south Africa[38]. These differences may be associated with the abundance and constancy of food availability, whereby agricultural-based cultures are associated with lower frequency of *APOE*-ε4; in this regard, *APOE*-ε4 has been hypothesized to be a "thrifty" allele that might offer an advantage in conditions of food scarcity[38]. Importantly, the *APOE*-ε4 allele has been shown to be the ancestral allele (i.e. the sequence found in the last common ancestor in humans;[39,40]).

A) B)



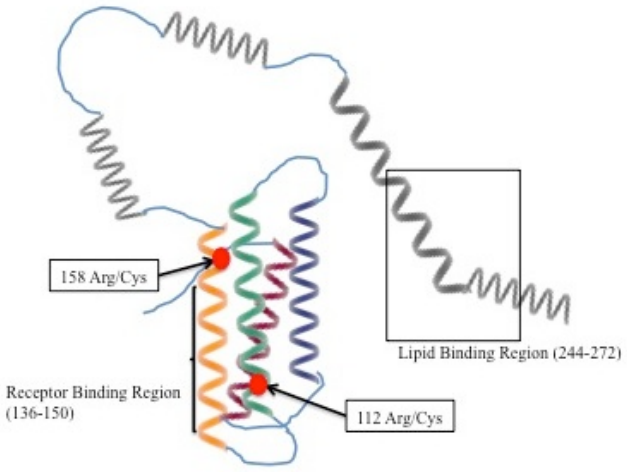


Figure 1.4 Apolipoprotein: primary and tertiary structure

A) Primary structure of ApoE and amino acid changes in positions 130 and 176 are highlighted. The ε2 allele has a cysteine at both positions, ε3 allele has a cysteine-130 and arginine-176, and the ε4 allele has arginine at both positions. The protein has a 34Kd molecular weight (retrieved from UniprotKB; prot ID P02649). NCBI database of single-nucleotide polymorphisms indicates the rs429358 alleles C/T, C being the ancestral coding for the Cys/Arg

at 130 and rs7412 alleles C/T, C being the ancestral coding for the Cys/Arg at 176. B) tertiary structure of folded apoE in it soluble form. Figure shows the lipid binding domain, the receptor binding domain as well as the position of the two Arg/Cys polymorphisms (adapted from[41]).

1.4.2 Apolipoprotein E (apoE)

ApoE is a 317aa arginine-rich protein containing an 18aa signal peptide that is intracellular cleaved before being secreted prior to glycosylation[37]. In its lipid-free form, apoE contains two distinct, independently folded structural domains with different functions. The amphipathic α-helix domain allows the protein to adopt a lipid-bound and a lipid-free state, whereas the helix 4 contains the receptor-binding sequence; see Figure 1.4[41]. The three main protein isoforms resulting from the allele polymorphisms differ by a cysteine-arginine interchange in one of two positions at residues 130 and 176[33–36]. The binding affinity of apoE is different for each the apoE isoforms. ApoE3 and apoE4 have similar binding affinity for Low-Density Lipoprotein receptor (LDLr) but apoE3 shows preferential binding to small High Density Lipoprotein (HDL)-like particles, whereas apoE4 shows preferential binding to larger, Very Low Density Lipoprotein (VLDL)-like particles; on the other hand apoE2 has a 100-fold weaker affinity to LDLr compared to apoE3 and apoE4[42]. ApoE also undergoes conformational changes when it is bound to lipids as opposed to its unbound-lipid state. When containing mainly phospholipids it tends to form discoid complexes containing four apoE molecules per disk with characteristics similar to HDL particles[43]. In contrast, when containing a core of triglycerides and phospholipids, it forms tetramer complexes resembling VLDL particles[44].

ApoE is synthesized most abundantly in the liver, and in the brain and adrenal glands (~30% relative to liver), but also in several other tissues (e.g. kidney and lungs, ~10% relative to

liver[45]. ApoE belongs to the group of proteins that form lipoproteins, which are particles constituted from the combination of lipids and various proteins. ApoE plays a critical role in cholesterol and triglyceride transport. ApoE is the main protein constituent of chylomicrons which are large, low-density particles that collect the dietary cholesterol and triglycerides absorbed in the digestive system prior to uptake by the liver, a process mediated by LDL receptor binding of apoE (exogenous pathway). Further, cholesterol and triglycerides are distributed from the liver to the rest of the cells in the body by VLDL and LDL particles where apoE is one of the main protein constituents (endogenous pathway). The metabolism of cholesterol also has a particular feature whereby redistribution of cholesterol from other parts of the body to the liver may occur. This metabolic pathway is better known as the reverse cholesterol transport, and is mediated by HDL particles where apoE is the main protein constituent. Exogenous and endogenous pathways as well as reverse cholesterol transport are summarized in Figure 1.5.

The brain does not rely on this systemic cholesterol metabolism and transport, but rather has its privileged, local, and auto- and paracrine-like cholesterol homeostasis system in which apoE plays a critical role. Indeed, in the brain, apoE is synthesized mostly by astrocytes, and to a lesser extent, by activated microglia and neurons during certain developmental stages or during repair[46].

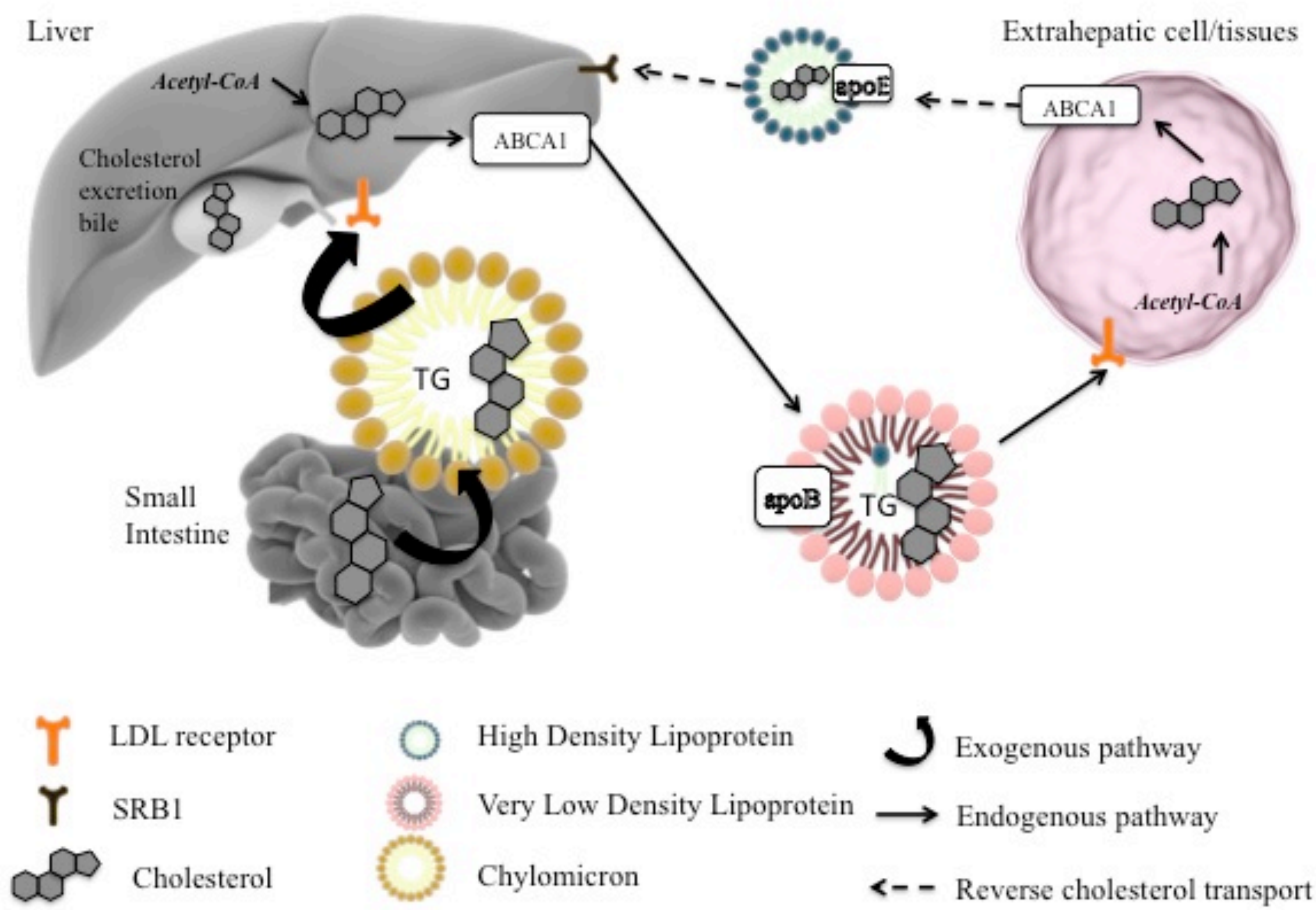


Figure 1.5 Cholesterol and triglyceride metabolism, including reverse cholesterol transport

([illegible][47])

Synthesis of apoE in the brain begins *in utero*, increases during development, and reaches a steady state in adulthood. The synthesis of apoE appears to occur in all brain neuroanatomical locations[45], predominantly in grey matter compared to white matter (described in Chapter 2). ApoE is the major apolipoprotein in the brain where it forms HDL-like particles that distribute cholesterol to neurons and their supporting cells[48–50]. In addition, apoE plays an important role in the metabolism of cholesterol, participating in its clearance outside of the brain. While nascent particles secreted by astrocytes barely contain core lipid and are discoid in shape, particles in the cerebral spinal fluid (CSF) are of the size and shape of HDL particles (spherical) and contain the core lipid as well as esterified cholesterol[51], see Figure 1.6.

1.4.3 ApoE function in the nervous system

Pioneering studies on peripheral nerve and optic nerve in animals identified *de novo* protein synthesis (~37Kd protein) 3 to 7 days following an injury to the nerve[52,53]. Subsequently, it was demonstrated that apoE was synthesized and secreted by astrocytes in the CNS as well as by Schwann cells associated with non-myelinated fibers in the peripheral nervous system[48,49]. These two lines of inquiry converged with the demonstration that apoE was indeed the protein synthesized by reactive macrophages after nerve injury[54–56].

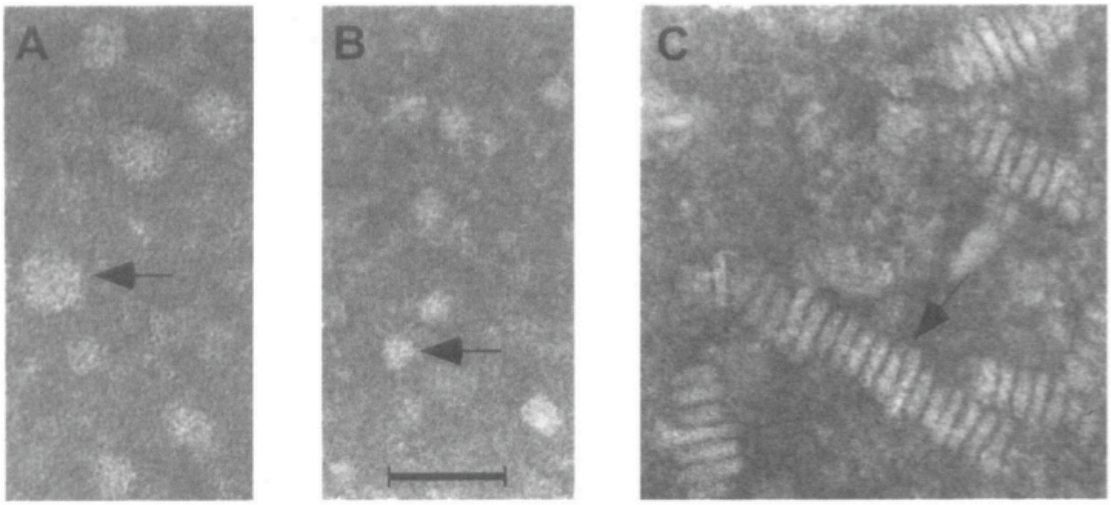


Figure 1.6 Negative-stained electron micrographs of lipoproteins

Panels A and B show spherical particles from two different fractions of CSF (in humans) and panel C shows stacked discoid particles from rat-astrocyte conditioned media. Size bar is 25nm. With permission from[51].

In addition, it was demonstrated that apoE is internalized by neuronal growth cones via LDLr binding and that apoE plays a key role in neurite outgrowth[57,58] . Furthermore, it was shown that when apoE3 (plus VLDL) was added to a neuron culture the neurite growth was optimal, whereas apoE4 (plus VLDL) inhibited neurite growth[58]. However, the effect of apoE was not limited to structural changes, but also to functional ones. Whereas incubating retinal ganglion cells in a astrocyte-free cultures enabled the formation of barely functional synaptic contacts, when cultured with astrocytes the synaptic activity increased 70-fold, barely any transmission failures occurred, and the action potential quanta release significantly increased[59].

The last piece of evidence to support the relevance of apoE function to synaptic plasticity, membrane homeostasis and repair came from demonstrating its crucial role in cholesterol metabolism in the brain to allow optimal synaptic function. Elegant experiments using cultured retinal ganglion cells showed that i) cholesterol alone, but not other lipids (e.g. phospholipids) increased synaptic activity; ii) inhibition of cholesterol production with a acetyl-CoA inhibitor shut down synaptic activity and this activity was rescued by adding cholesterol to cultures; iii) blocking the LDLr receptor reduced synaptic activity and iv) the maximum level of synaptic activity was obtained when optimal levels of **both** apoE and cholesterol were present[60]. ApoE is now recognized as the essential vehicle transporting and helping to regulate cholesterol availability, and cholesterol is a necessary component to synapse and membrane homeostasis in the brain. The central role of apoE and its intricate partnership with cholesterol is shown in Figure 1.7.

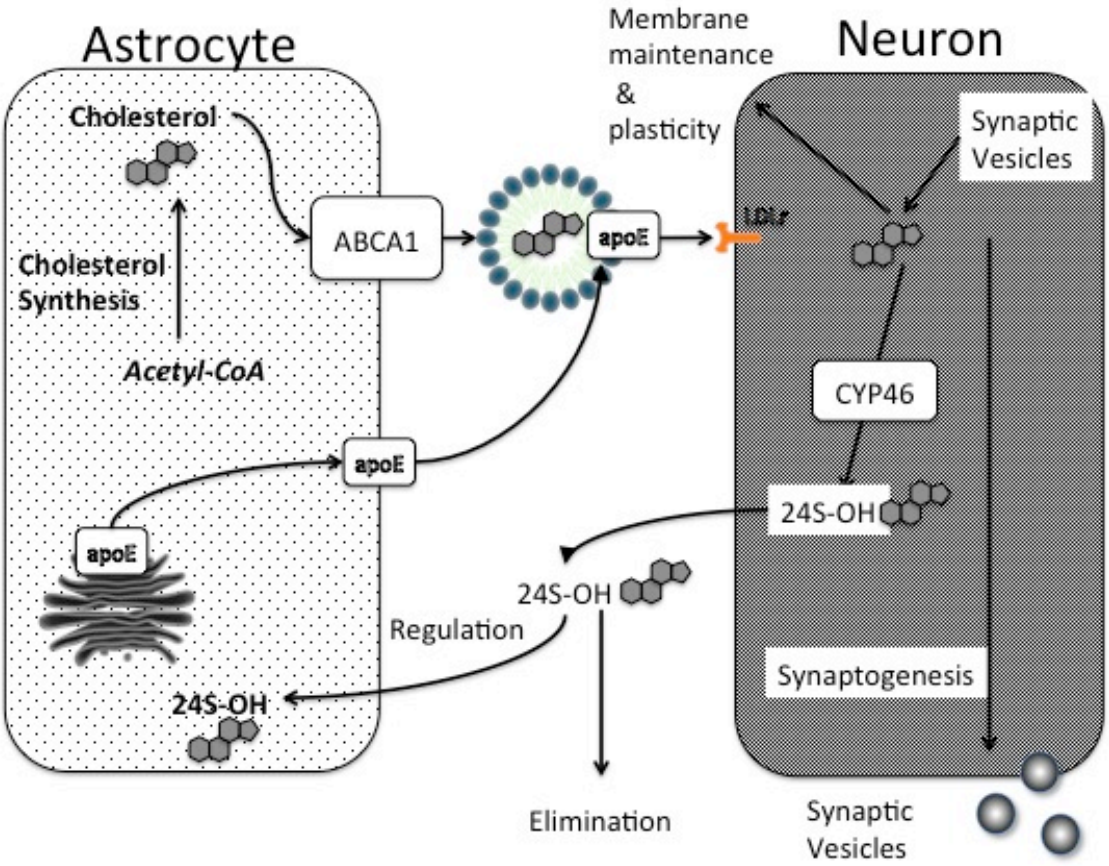


Figure 1.7 ApoE and cholesterol metabolism in the brain

Astrocytes synthesize and secrete apoE; ABCA1 cholesterol transporter excretes cholesterol synthesized in astrocytes into apoE. ApoE transports the cholesterol to neurons via LDLr binding. Cholesterol is utilized for membrane maintenance, synaptogenesis, and synaptic transmission. 24S-hydroxycholesterol (24S-OH) is secreted (CSF and plasma), and also regulates cholesterol synthesis by astrocytes[48,51,61–63]. Figure modified from Bjorkhem and Meaney[61].

1.4.4 Apolipoprotein E varepsilon 4 in disease states

Shortly after the discovery of apoE's role in brain cholesterol homeostasis, neurite outgrowth, synaptogenesis, and neuronal plasticity, apoE was found to be the protein binding to amyloid beta (Aβ), the main component of amyloid plaques in the brain[64,65], which are a neuropathological hallmark of Alzheimer's disease (AD) dementia. This finding prompted researchers to investigate the role of *APOE* in AD and to discover that *APOE*-ε4 is the main genetic risk factor for developing late-onset AD[66–69]. A number of studies have demonstrated that carrying an *APOE*-ε*4* allele worsens the prognostic outcome in a wide variety of brain illnesses such as traumatic brain injury, cerebral hemorrhagic stroke, and Korsakoff's psychosis[70–72]. The evidence suggests that the presence of the *APOE*-ε4 variant is related to an impaired capacity for recovery from brain insults.

Although the precise pathophysiological mechanism by which *APOE*-ε4/apoE4 leads to AD has not been fully elucidated, it has been shown that the cys112arg substitution in apoE4 leads to a tertiary and quaternary structure that is more prone to be in a conformational state that has a different folded molecular structure of the protein[41]. This makes apoE4 more prone to aggregation and degradation[73], results in impairments to its cholesterol delivery role by a difference in affinity binding to LDLr based on size of HDL particles[42], and affects its capacity to provide neurotrophic signal[74]. In addition, peptide fragments from apoE4 have been shown to have a neurotoxic effect[75].

1.5 Apolipoprotein E and Schizophrenia

Two years after the association of *APOE*-ε4 and AD was reported, Harrington and colleagues investigated the frequency of *APOE* alleles in post-mortem brain tissue from 165

patients with AD, 42 patients with SCZ, and 131 age- and sex-matched controls. The frequency of the *APOE*-ε4 allele was 32% in AD, 26% in SCZ, and 15% in controls. The frequency of *APOE*-ε4 was significantly higher in AD and SCZ compared to controls, but similar between AD and SCZ. Further analyses splitting the SCZ sample into "young" and "old" based on age at time of death (i.e. below or above 70 years old, respectively), showed no statistically significant difference in frequency of *APOE*-ε4 allele in the two groups. The authors inferred that the increased frequency of *APOE*-ε4 was unlikely to be driven by the co-existence of AD-like pathology in SCZ in their sample[76].

The study by Harrington and colleagues[76] has been followed by extensive research on the role of *APOE*/apoE in SCZ (see Table 1.2 for summary of literature). Research has focused mostly on the question of whether there is an association between *APOE* allele frequency and SCZ, particularly the *APOE*-ε4 allele. In addition, studies have explored whether *APOE* alleles modify or mediate aspects of the clinical phenotype such as age of onset, response to treatment, or severity of clinical presentation (Table 1.2; A). A second main area of interest has been on proteomic, molecular, and neuropathological aspects of *APOE*/apoE in SCZ (Table 1.2; B).

A. Studies ascertaining the frequency of *APOE* alleles in patients with SCZ and associated clinical phenotypes (chronological order)

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[77]	Y	Cross-sectional	87 SCZ; 57 CTRL	Cau	No difference	No association of *APOE*-ε4 with age of onset	
[78]*	Y	Cross-sectional; IP	98 SCZ; 98 CTRL	Cau	No difference	No association of *APOE*-ε4 with age of onset; *APOE*-ε4 less positive symptoms, in [79]	
[80]	Y	Cross-sectional	51 SCZ; 35 CTRL	Cau	No difference	No association of *APOE*-ε4 with IQ	
[81]*	Y	Cross-sectional; IP	122 SCZ; 126 CTRL	Jap	No difference	No association of *APOE*-ε4 with age of onset	
[82]	N	Cross-sectional	52 SCZ	Cau	No difference	*APOE*-ε4 lower BPRS scores in drug-free* SCZ	
[83]	Y	Cross-sectional; inpatient	62 SCZ; 63 CTRL	Tai	No difference		
[84]*	Y	Cross-sectional	164 SCZ; 141 CTRL	Jap	*APOE*-ε2 less frequent in SCZ		
[85]	Y	Cross-sectional	63 SCZ; 301 CTRL	Cau	No difference		
[86]*	Y	Cross-sectional; OP/IP	54 SCZ; 43 CTRL	Jap	No difference	*APOE*-ε4 less frequent in early onset	

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[87]	N	Cross-sectional	87 SCZ	Jap	NA	No association of *APOE*-ε4 with age of onset, symptoms.	
[88]	Cross-sectional	18 SCZs	Cau	NA		*APOE*-ε4 attenuates ketamine-induced psychosis in SCZ	
[89]	N	Cross-sectional; IP	144 SCZ; 134 CTRL	Jap	No difference (reg regions)		
[90]	Y	Cross-sectional	69 SCZ; 140 AD; 121 CTRL	Cau	No difference		No association of *APOE*-ε4 with age of onset
[91]*	Y	Cross-sectional	134 SCZ; 103 CTRL	Cau	No difference		
[92]*	Y	Cross-sectional	106 SCZ; 98 CTRL	Cau	No difference	No difference in deficit vs. non-deficit SCZ	
[93]	Y	Prospective; inpatient/outpatient	40 COS; 57 CTRL	Cau	No difference	No association of *APOE*-ε4 with age of onset, symptoms, or hippocampal volume	
[94]	Y	Cross-sectional; inpatient	237 SCZ; 137 CTRL	Tai	*APOE*-ε3 increased in SCZ; *APOE*-ε2/ε4 decreased in SCZ		
[95]*	Y	Cross-sectional; IP	333 SCZ; 191 CTRL	Jap	No difference		*APOE*-ε4 is higher in men with tardive dyskinesia

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[96]	Y	Cross-sectional	114 SCZ; 94 CTRL	Cau	No difference	*APOE* ε4 trend associated with earlier onset	
[97]	N	Cross-sectional; IP	95 SCZ	Chi	NA	No difference in CLZ response or other variables for *APOE*-ε4 carriers	
[98]*	Y	Cross-sectional; IP	314 SCZ; 188 CTRL	Jap	No difference		
[99]	Y	Cross-sectional; IP	60 SCZ; 60 CTRL	Kor	*APOE*-ε3 increased in SCZ; *APOE*-ε2 decreased in SCZ		
[100]	Y	Cross-sectional; OP	365 SCZ; 584 CTRL	Cau	No difference	*APOE*-ε4 associated with earlier onset and worse negative symptoms in women	
[101]	N	Cross-sectional; OP	21 SCZ	Jap	NA		No difference in hippocampal volume in *APOE-ε4*
[102]	Y	Cross-sectional	106 SCZ; 250 CTRL	Cau	No difference	No association to age of onset	
[103]	Y	Cross-sectional; OP/IP	579 SCZ; 1528 CTRL	Chi	*APOE*-ε4 increased in SCZ	Increase of risk associated with periods of famine	
[104]	Y	Cross-sectional; OP/IP	114 SCZ; 91 CTRL	Cau	No difference		

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[105]	Y	Cross-sectional	94 SCZ; 98 CTRL	Cau	No difference	*APOE*-ε4 associated with earlier onset	No association to treatment response
[106]	Y	Cross-sectional	28 SCZ; 24 CTRL	Cau	NR	Impaired olfaction not associated with *APOE*-ε4	
[107]	N	Cross-sectional	35 SCZ, 36 CTRL	Chi	NA		apoE did not change with CPZ; apoE decreased in CSF in medicated SCZ
[108]	N	Cross-sectional; IP	86 SCZ (Of 97)	59% Afr; 41% Cau	NA		*APOE*-ε2 more frequent in Caucasian TD
[109]	Y	Cross-sectional; outpatients	60 SCZ; 42 CTRL	Mex	No difference		
[110]	N	Cross-sectional; OP/IP	585 SCZ; 615 CTRL	Cau	No difference		
[111]	N	Prospective; OP	28 SCZs; 23 CTRL	Cau	NA		ApoE decreased in plasma drug-naïve SCZ;
[112]	N	Cross-sectional	427 SCZ	Cau	NA		*APOE*-ε4 associated with diabetes
[113]	N	Cross-sectional; outpatients	60 pairs	Mex	NA		Association in the presence of linkage for *APOE*-ε3 and SCZ

							in females
Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[114]	Y	Cross-sectional; outpatients	207 SCZ; 165 CTRL	Ara	*APOE*-ε2 less frequent in SCZ	*APOE*-ε4 less frequent late onset	
[115]	N	Cross-sectional; inpatient	17 FES; 10 CTRL	Cau	NR	Up regulation of apoE in SCZ after mean of 13 days of treatment; no correlation of apoE with other clinical/demographic variables.	
[116]	Y	Cross-sectional	76 SCZ; 82 CTRL	Cau	No difference		
[117]	N	Cross-sectional	336 SCZ; 172 CTRL	Cau	No difference		
[117]	N	Cross-sectional	308 SCZ; 129 CTRL	NR	No difference		*APOE*, APOER2, VLDLR associated with premorbid IQ and verbal memory
[118]	N	Prospective; inpatient/outpatient	129 SCZ	Tai	NR		*APOE*-ε4 associated with total cholesterol; No change of lipid profiles after Paliperidone for any *APOE* allele
[119]	N	Cross-sectional; outpatients	180 SCZ; 200 CTRL	Ara	*APOE*-ε2 more frequent in SCZ	*APOE*-ε2 associated with younger age of onset	

[120]	N	Cross-sectional	711 SCZ; 665 CTRL	Tai	No difference		
[121]	N	Cross-sectional; outpatients	122 SCZs	45% Cau	No difference	*APOE*-ε4 lower verbal memory only in low-education group	

B. Studies in post-mortem brain samples ascertaining proteomic, molecular, and neuropath logical aspects.

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[122]*	N	Cross-sectional: PM	35 SCZ, 35 CTRL	Cau 95%	No difference		Reelin mRNA correlated to apoE
[123] *	Y	Cross-sectional: PM	35 SCZ, 35 CTRL	Cau 95%	No difference	ApoE in BA9 no different in SCZ; *APOE*-ε2 higher apoE	
[124]	N	Cross-sectional; PM	15 FES; 15 CTRL	Cau	NR	LRP10 and apoE increased, and LRP12 decreased in FES	
[125]	N	Cross-sectional; PM	110 SCZ	Cau 76%; Afr 19%	NA	*APOE*-ε4 associated with NP, but not FT in hippocampus	
[126]*	N	Cross-sectional; PM	19 SCZ; 18 CTRL	Cau	NA	*ApoE increased in BA9 and BA46 in SCZ	
[127]*	Y	Cross-sectional; PM	23 SCZ; 47 CTRL	Cau	No difference	*ApoE increased in BA9 in SCZ	
[128]	N	Cross-sectional; IP; PM	116 SCZ; 172 AD; 19 CTRL	NR	*APOE*-ε4 more frequent in SCZ+AD	AD and SCZ+AD had similar frequency of *APOE*-ε4; SCZ no AD similar *APOE*-ε4 than CTRL	

Reference	Meta	Type of Study	Sample Size	Ethnicity	Allele Frequency	Clinical Phenotype	Other
[129]	Y	Cross-sectional; PM	175 SCZ; 39 LBD; 50 PD; 400 CTRL	Cau	No difference	*APOE*-ε4 more frequent in LDB and AD	
[130]	Y	Cross-sectional;OP/PM	93 SCZ; 106 CTRL	Cau 60%; Afr-Am 40%	No difference	*APOE*-ε4 more NFT, but not AP	
[131]	N	Cross-sectional; PM	9 SCZ; 9 SCZ; 62 Dem; 243 CTRL	Cau	SCZ+Dem, lowest frequency of *APOE*-ε4,	AD +/- Lewy Body pathology highest frequency of *APOE*-ε4	
[76]	Y	Cross-sectional; PM	42 SCZ; 165 AD; 131 CTRL	Cau	*APOE*-ε4 more frequent in SCZ		
[132]	N	Cross-sectional; OP	23 Paraph; (20 women)	Cau	*APOE*-ε4 less frequent in paraphrenia		

CTRL = control; SCZ = schizophrenia; OP = outpatient; IP = inpatient; PM = post-mortem; AD = Alzheimer's disease; Dem = dementia; LBD = Lewy-body dementia; PD = Parkinson's disease; NR = not reported; Afr-Am = African-American; Ara = Arabic; Cau = Caucasian; Chi = Chinese; Jap = Japanese; Kor = Korean; Mex = Mexican; Tai = Taiwanese;

Table 1.2 Summary of studies on apolipoprotein E in schizophrenia

1.5.1 *APOE* alleles and association to schizophrenia

Five independent meta-analyses have addressed the question of whether different *APOE* alleles are associated with increased risk of SCZ[104,109,133–135]. The meta-analyses are summarized in Table 1.3[g].

The most salient commonality amongst these five meta-analyses is the absence of a clear pattern of association between *APOE* alleles and SCZ. The findings to date are mostly non-convergent and often directly contradictory. Only one meta-analysis found an increased risk of SCZ associated with *APOE*-ε4 status, and this finding was only significant when studies including Caucasian samples were included[134]. In contrast, three other meta-analyses did not find an increased risk of SCZ associated with *APOE*-ε4 status[109,133,135]. Schürhoff and colleagues actually reported a *protective* effect of *APOE*-ε4 against SCZ in Asian populations only[104]. Additionally, *APOE*-ε3 status has been reported to be both a protective[133] and a risk factor for SCZ in Asian populations[104]. Although it could be argued that the most recent meta-analysis by Gonzalez-Castro et al. that includes seven studies provides the most reliable evidence regarding the true effect of the association of *APOE*-ε3 to SCZ (e.g. the meta-analysis by Schürhoff and colleagues included only three studies), it should be noted that four of seven studies included in

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[g] Each of the corresponding authors of these studies was contacted to comment on whether their samples overlapped (no responses received). Of note, the meta-analysis by Allen and colleagues denotes the studies marked with an asterisk as overlapping (supplementary Figure 2.1, Allen et al., 2008).

the meta-analysis by Gonzalez-Castro and colleagues are likely to include overlapping, non-independent samples[81,91,92,95].

Reference	Number Studies	Sample Size	Risk	Conclusion
[133]	28 (7)	3452 SCZ; 4703 CTRL	ε3 OR = 0.73; CI (0.54–0.98)	No association ε4-SCZ; ε3 protective (Asian)
[109]	22	2810 SCZ; 4197 CTRL	ε4 OR = 1.06; CI (0.89-1.27), p = 0.1586	No association ε4-SCZ
[134]	24 (15)	1500 SCZ; 2702 CTRL	ε4 OR = 1.16 CI (1.00–1.34); p = 0.043 (Cau)	ε4 associated with SCZ (Caucasian)
[135]	17	2194 SCZ; 2615 CTRL	ε4 OR = 1.08; CI (0.88–1.33), p = 0.45	No association ε4-SCZ
[104]	14 (3)	1949 SCZ; 2354 CTRL	ε4 CI (0.92–1.22), p = 0.38; ε3 OR = 1.53; CI (1.2–1.97); p = 0.0007 (Asian); ε4 CI (0.50–0.95); p = 0.02 (Asian)	No association ε4-SCZ; ε4 protective (Asian); ε3 associated with SCZ (Asian)

In brackets the number of studies used for sub-analyses on particular population; SCZ = schizophrenia, CTRL = control, OR = odds ratio, CI = 95% Confidence Interval.

Table 1.3 Summary of meta-analyses on association of *APOE* alleles and schizophrenia

Two critical aspects based on this systematic review of the literature concerning *APOE* allele frequency in SCZ are noted: i) the potential risk that *APOE* allele(s) might confer would be of small magnitude similar to many other genes associated with SCZ[134], and ii) the risk associated with *APOE* allele(s) in SCZ may differ based on an array of additional variables such sex[100], ethnicity[99], or even interactions of biological and environmental factors such as periods of famine in Asian populations[103]. This suggests that *APOE* alleles may be associated with pleiotropic effects.

Similar to *APOE*, additional genetic risk factors in SCZ have been shown to have a small effect size. A plausible explanation may be related to the fact that most research studies use the DSM (or the ICD) as their diagnostic tool and, as discussed above, this diagnosis is *de facto* a syndromic diagnosis. It is plausible to hypothesize that our current diagnosis of SCZ may be grouping separate nosological entities, and that separate nosological entities may be associated with a specific genetic risk factor (e.g. "schizophrenia disease 1" → gene A; "schizophrenia disease 2" → gene B, etc.). In support of this hypothesis, data from genetic studies of families at high risk for SCZ result in larger effects sizes as they group samples not only on the syndromic diagnosis, but also on a common pool of genetic risk[136]. Importantly, we would be able to test this hypothesis directly as soon as research designs begin to incorporate biomarkers to classify patients rather than just using consensus diagnostic criteria[137].

Regarding *APOE* pleiotropic effects, it is important to take into account existing evidence emerging from the study of cardiovascular disease (CAD) and AD. African populations, particularly sub-Saharan populations, are characterized by a higher prevalence of *APOE*-ε4 which can be as high as ~ 40% in Digmes, Khoi San, or Yoruba[138–140]. However, total and LDL

cholesterol levels in these sub-Saharan populations are similar to those found in *APOE*-ε3 homozygotes, and this is in contrast to African-American populations with high prevalence of *APOE*-ε4 allele where total and LDL cholesterol levels are much higher[141–143]. In addition, the prevalence of AD in elderly sub-Saharan populations with a high prevalence of *APOE*-ε4 allele is similar to that of *APOE*-ε3 homozygotes, as opposed to the risk association of *APOE*-ε4 to AD in African-American populations[144–147]. The risk associated with *APOE*-ε4 in African-American individuals appears to be weaker than their Caucasian *APOE*-ε4 carrier counterparts[146,148].

It is posited that the heterogeneity of results in terms of genetic association of *APOE* and SCZ, and even contradictory evidence, not only emerges from methodological differences, but also from true pleiotropic effects of *APOE* resulting from gene by environment interactions. The differences observed in the clinical phenotype and *APOE*-ε4 allele in SCZ may provide further evidence of pleiotropic effects of *APOE* on SCZ.

1.5.2 *APOE* alleles and clinical phenotype

Several research groups have explored putative relationships between *APOE* alleles and different aspects of the clinical presentation in SCZ, and results have been heterogeneous. Pickar and colleagues investigated *APOE* genotypes and measured severity of positive psychotic symptoms using the Brief Psychiatric Rating Scale (BPRS) in a group of chronic SCZ patients during a neuroleptic-free period (mean duration ~ 27 days). They compared patients with at least one *APOE*-ε4 allele to those without an *APOE*-ε4 allele. In *APOE*-ε4 carriers, BPRS psychosis scores were lower during a drug-free period despite having similar age at index admission as well as duration of the neuroleptic-free period[82]. Interestingly, a subgroup of these patients received in a crossover design intravenous ketamine (0.12 mg/kg bolus and 0.65 mg/kg infusion or placebo over 1h). They found *APOE*-ε4 carriers displayed an attenuated ketamine-induced

psychotic symptom response as measured using BPRS[88]. However, these results are divergent from those by Rietschel and colleagues who used the Operational Criteria Checklist for Psychotic Illness and Affective Illness (OPCRIT) in a sample of patients with chronic SCZ who were stable. They reported *APOE*-ε4 carriers had more severe positive symptoms such as "incoherence", "speech difficult to understand", and "persecutory delusions" compared to those who were not *APOE*-ε4 carriers[79]. They argued in a response to the report by Pickar et al. that the apparent discrepant results might have been due to methodological differences in the two studies. Rietschel and colleagues posed that the OPCRIT documented the presence or absence of symptoms during a lifetime as opposed to the acute state symptoms measured by the BPRS. This suggested their study might have detected a trait difference whereas the study by Pickar and colleagues might have detected a state difference (of opposite direction). Another significant difference between the two studies was the fact that patients in Pickar et al. were assessed during a neuroleptic-free period whereas those in Rietschel et al. were assessed while on treatment and may provide support for a pleiotropic effect of *APOE* mediated by medications.

Ward and colleagues also explored the relationship of *APOE*-ε4 allele with symptom domains, particularly cognitive impairment (verbal memory). Using the Brief Assessment of Cognition in Schizophrenia (BACS) scale, they assessed verbal memory in middle-aged chronic SCZ patients on stable pharmacological regime for 6 months (mean age ~ 43 years old). They reported that those who were *APOE*-ε4 carriers had worse verbal memory performance only if they were in the low-education range of the sample, suggesting again an *APOE*-ε4 by environment interaction in SCZ, and potentially a pleiotropic effect that varies with level of education[121].

In addition, pleiotropic effects are not limited to positive symptoms and cognitive impairment, but may also extend to pleiotropic effects associated with sex and age of onset. A study in a large sample of Spanish chronic SCZ patients on stable pharmacological regime reported an association of *APOE*-ε4 with early age of onset and worse negative symptoms, but only in women patients[100]. The authors hypothesized an interaction of *APOE*-ε4 with hormonal factors, which may be associated with the relationship between *APOE*-ε4 and fertility rates[149,150]. *APOE*-ε4 has been shown to increase the fitness to reproduce in humans, or to have a beneficial effect in women during reproductive age (i.e. evolutionarily advantageous). Convergent evidence of an association between *APOE*-ε4 and earlier age of onset in SCZ was reported in several other studies with smaller sample sizes[96,105,130]. Given the findings from Martorell and colleagues, it can be speculated that the signal observed in these additional reports was driven by an earlier age of onset in *APOE*-ε4 women as these studies had a lower frequency of males than would be expected in a representative sample in SCZ (43% males in Durany et al.[96]; 49% males in Kampman et al.[105]; and 54% males in Arnold et al.[130]). Convergent with this hypothesis, the larger proportion of men may account for the negative findings regarding the association of *APOE*-ε4 to age of onset in other studies where the proportion of men was larger (e.g. 57% males in Sáiz et al.[102], 59% males in Ohara et al.[87], 62% males in Zhu et al.[151], and 67% males in Thibaut et al.[91]. In addition, two other studies in an Arabic sample[119], and a Korean sample[99] have reported an association of *APOE*-ε2 allele and SCZ (SCZ) and with earlier age of onset. However, a separate study in an independent Arabic sample found *APOE*-ε2 carriers to be significantly less frequent in SCZ compared to controls[114]. The low prevalence of *APOE*-ε2 combined with small sample size might explain these divergent findings.

Last, three studies investigated the association of *APOE*-ε4 allele to treatment response to neuroleptics. Whereas one study specifically examined whether *APOE*-ε4 was associated with clozapine response[97], a second one explored *APOE*-ε4 association to typical neuroleptic response[116], and lastly, Durany and colleagues did not report the type of neuroleptics their study patients were on[96]. None of these studies found an association between *APOE*-ε4 and treatment response.

1.5.2.1 Neuropathology, proteomics, and molecular biology of *APOE*/apoE in schizophrenia

Alois Alzheimer himself was among those who pioneered research into the neuropathology of SCZ (for him at the time, dementia praecox), and [unknowingly] *APOE*. In a subgroup of patients with SCZ, he described neuropathological findings in the cortex similar to those he observed in dementia (later coined after his name), but of lesser intensity and in particular, with less glial reactivity[152,153]. Whether SCZ was a progressive neurodegenerative disorder with similar neuropathology to that of other dementias was the object of inquiry for part of the second half of the 20th century. This body of research is summarized by Baldessarini in a systematic review and meta-analysis on the topic[154]. The main conclusion was that neurite plaques and neurofibrillary tangles were not found in higher frequency in elderly SCZ patients compared to age-matched controls. These data, in addition to accruing support for a neurodevelopmental etiopathogenic component in SCZ, appeared conclusive. The finding of association of the *APOE*-ε4 allele to AD sparked renewed interest in the topic of the underlying neuropathological signature of SCZ and whether this particular genetic risk factor was the one associated with neuropathological findings. Two studies adopted the strategy of determining the frequency of *APOE*-ε4 in several forms of dementia as well as in SCZ patients with or without

dementia processes[129,131]. Martinoli and colleagues had access to a sample of 243 controls, 71 dementia cases of different types [with or without Parkinson's disease (PD); with or without Lewy body disease (LBD)] and nine SCZ cases with dementia. *APOE* was genotyped for all cases and the only group with a higher *APOE*-ε4 frequency than controls was AD[131]. This study had limited statistical power given the small subgroup samples sizes and the attendant broad confidence intervals of these samples. In contrast, St. Clair collected a very large sample of Caucasian post-mortem human brain tissue from 175 patients with SCZ, 39 LBD, 50 PD, 153 AD, and 400 controls and found that *APOE*-ε4 allele frequency was only increased in LBD and AD. The frequency of *APOE*-ε4 in SCZ was 15% the same frequency reported for the controls[129]. Arnold and colleagues adopted a different strategy and took one step further by analyzing in detail the presence of neurofibrillary tangles (NFT) and amyloid plaques (AP) and their association to *APOE*-ε4 in subsample of nineteen human brains from patients with SCZ[130]. They found those who were *APOE*-ε4 carriers had higher ratings of NFT and there was a trend for a correlation between the number of *APOE*-ε4 alleles and the number of NFT ($r = 0.44$, $p = 0.06$). In their sample, there was no association between AP and *APOE*-ε4 allele[130], and they did not find any case where enough neuropathology was present to justify a formal diagnosis of a neurodegenerative process. Although these results are convergent with the majority of the literature[154,155], they are in contrast to other reports in larger sample-size studies which indicate a higher prevalence of AD-like pathology in SCZ than in the general population[156]. Although in a follow-up study, this same group reported a lower prevalence of definite neuropathologically-defined criteria for AD in SCZ of 8%[157]. Interestingly, in a follow-up study Arnold and colleagues found a correlation between cognitive impairment and NFT/AP in non-demented SCZ patients, and those SCZ patients who did not show definite cognitive impairment had

significantly fewer NFT/AP than controls[157]. Powchik and colleagues further demonstrated that *APOE*-ε4 allele frequency in those with SCZ *and* AD was similar to those with AD only, but not to patients with SCZ only or those meeting criteria for other forms of dementia[128]. The latest piece in this puzzle came from a study by Rapp and colleagues, where they ascertained the presence of NFT and AP in five different brain regions, including the hippocampus, in post-mortem brain samples from patients with SCZ for whom a neurodegenerative condition had been ruled out. In this sample, they showed *APOE*-ε4 was strongly associated with the presence of hippocampal NFT and AP and that these neuropathological findings correlated with severity of cognitive impairment[125].

In summary, the existing evidence clearly demonstrates that in spite of progression of symptomology over the course of the disorder, there is no neuropathologically defined neurodegenerative process in SCZ. In addition, SCZ patients do not seem to be at higher risk of developing comorbid dementia processes, but there seems to be an association between genotype (*APOE*-ε4), neuropathology (NFT/AP), and symptoms (cognitive impairment) in SCZ (in the absence of a formal dementia diagnosis).

In terms of proteomic studies of apoE in SCZ, there have been only two prior instances investigating the abundance of apoE in post-mortem human brain samples of patients with SCZ[126,127]. Both studies investigated protein abundance in Brodmann area 9 (BA9), and one extended investigations to Brodmann areas 10, 40, 46, and caudate putamen[126]. In all studies there was no correlation between age or medication dose at time of death and amounts of protein in the brain. Dean et al.[126] and Digney et al.[127] found increased amounts of apoE in SCZ in BA9 (Digney et al. also found increased apoE in BA46). It is important to note that the Dean et al. and

Digney et al. studies did not use independent samples and the estimated overlap based on their sample description is likely more than 50%.

The expression of apoE outside the brain in SCZ was also studied in plasma and CSF. Dean and colleagues found that plasma apoE levels were decreased in drug-naïve SCZ patients compared to controls[111], but apoE was found to be up regulated in a first-episode SCZ sample who had been treated with second generation antipsychotics for a mean of 13 days[115]. In contrast, plasma apoE did not change after 8 weeks of chlorpromazine treatment in an independent sample[107]. Albeit preliminary, and only in small sample-size studies, these results indicate that changes in cholesterol and lipid metabolism are a promising area of future inquiry, as changes in lipid composition may be treatment related[158,159]. A remaining complex and unresolved question is to what extent changes in cholesterol metabolism in the periphery translate into changes in cholesterol metabolism in the CNS. Post-mortem human brain studies offer valuable, yet very limited information, and do not allow investigation of dynamic changes. Although the study by Wan and colleagues, which showed decreased levels of apoE in CSF in medicated SCZ patients compared to controls points to an effect of neuroleptics on brain cholesterol metabolism[107]. This suggests a potential pharmacodynamic link between neuroleptics and cholesterol metabolism in the brain related to the capacity of antipsychotics to up-regulate the sterol regulatory element-binding protein (SREB) and influence cholesterol and lipid metabolism[160–162].

Adjunct research investigating molecular biological and metabolomics aspects associated with *APOE*/apoE in SCZ has been conducted to investigate the relationship between *APOE*, apoE, cholesterol, reelin expression, and other lipids[122,123]. In addition, the expression of apoE-affine receptors, including low-density lipoprotein receptor-related protein 10 (LRP10) (increased expression) and LRP12 (decreased expression) in post-mortem BA46 samples of

recent onset SCZ patients (less than 4 years) was noted in cases where increased brain apoE levels where demonstrated[124]. It was argued that a differential pattern of molecular abnormalities exists at different stages of the illness, with altered lipoprotein metabolism/function being present in early stages[163]. Considering the impact of antipsychotics on cholesterol and lipid metabolism, a potential mechanism underlying the findings of Gibbons et al. (2010) would be that changes in early stages of the disorder are mediated by medications. Yet, they did not find any correlation between medication doses and expression of LRP10, LRP12 or apoE. In particular, when they looked only at patients who were on clozapine, which is associated with profound and marked metabolic effects, they found no association between the dose of clozapine and expression of the aforementioned proteins. The results of Kao and colleagues showing that *APOE*-ε4 had a significant positive impact on cholesterol and lipid changes (reduction of cholesterol and triglycerides) after initiation of treatment with paliperidone adds further evidence for pleiotropic effects of *APOE*-ε4 allele[118]. Finally, Verbrugghe and colleagues demonstrated in a large sample that *APOE*, *APOER2*, and *VLDLR* were associated with clinical outcomes, verbal memory, and premorbid IQ (controlling for medication effects), providing additional support for a primary involvement of cholesterol and lipid metabolism in SCZ[117].

1.6 Hippocampal formation and the schizophrenia syndrome

1.6.1.1 Hippocampus fundamentals

The hippocampus[h] is a bilateral structure of the brain located medially in the temporal lobe, underneath the floor of the temporal horn of the lateral ventricles (Figure 1.8). The hippocampus is part of the limbic[i] system along with the amygdala formation, the mammillary bodies, the fornix, and the cingulate gyrus[164,165]. The hippocampal formation is composed of the dentate gyrus, the hippocampus proper, the subiculum, and the entorhinal cortex. The hippocampus is considered grey matter due to its dense composition of cell bodies; it is considered allocortex/archicortex as it only contains three layers and phylogenetically is more primitive than the isocortex/neocortex[166].

Histologically (Figure 1.8), the hippocampus proper is subdivided into four areas: Cornu Amonis 1 to 4 (CA1 to 4), and is composed of three main layers: polymorphic (composed mostly of glia and interneurons), molecular (composed of a rich network of connections) and pyramidal (where cell somata of pyramidal cells are located). The dentate gyrus and the subiculum are both similarly composed of a polymorphic layer rich in interneurons and glia, a molecular layer with an extensive network of connections, and a granular layer densely packed with pyramidal neurons[167].

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[h] Etymologically, the hippocampus originates from Greek and means "sea horse", as its shape when dissected out from the rest of the brain is reminiscent of this organism.

[i] Etymologically, from Latin '*limbus*', meaning edge, boundary. This descriptive term was adopted since the limbic system forms a rim in the medial aspect of the cortex in the temporal lobe.

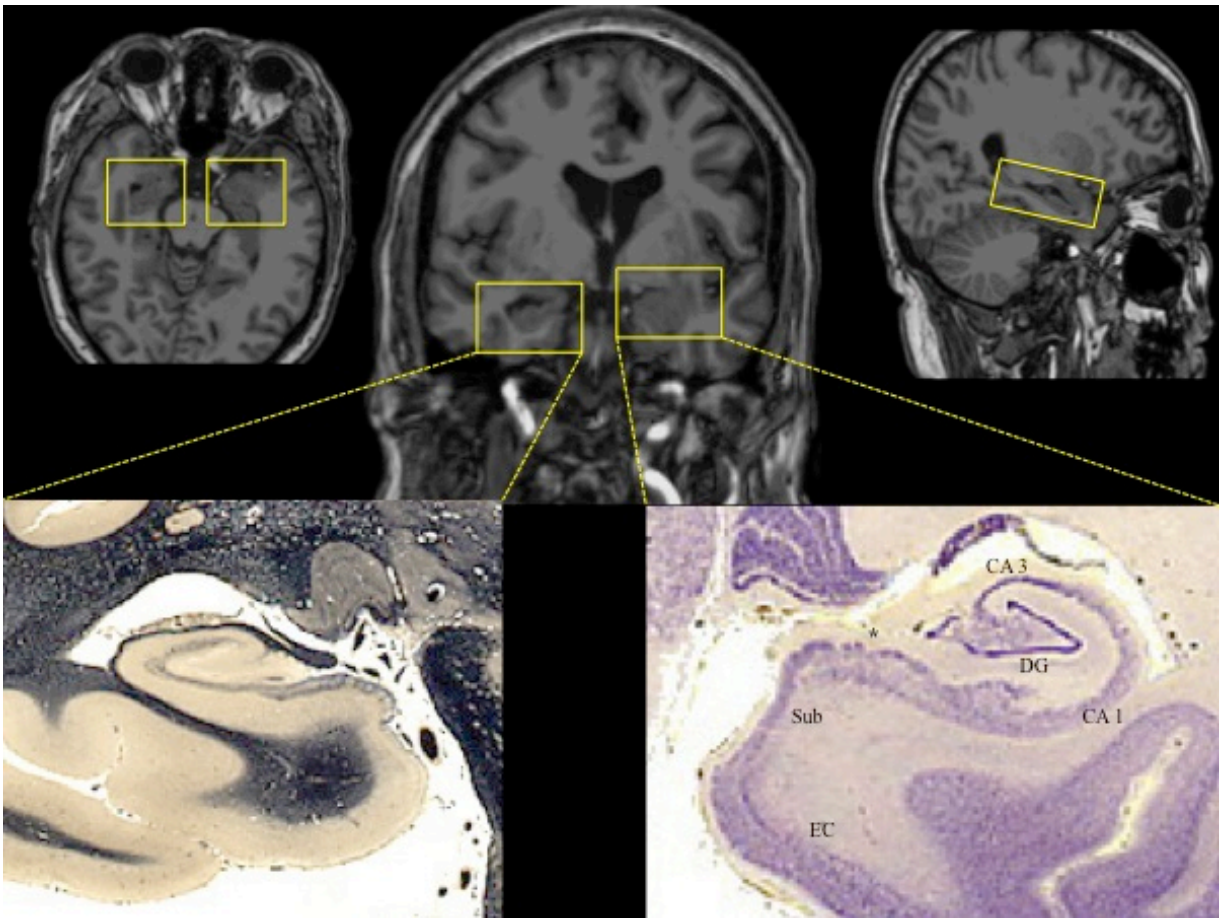

Figure 1.8 Hippocampus: anatomy and histological organization

In this figure the location of the hippocampus on a brain MRI is shown at the top of the figure circumscribed by yellow boxes (own data). From left to right in axial, coronal and sagittal planes respectively. The bottom sections are histological preparations of coronal hippocampal slices plane in a myelin stain (left) and hematoxilin-eosin staining for cell bodies (right). Note hippocampal subfields are noted on the right-hand side staining (histological images from the Michigan State University Human brain atlas, with permission).

The hippocampus has a very complex geometry that originates from its inward folding during embryonic development. Until week 15-16 of gestational age the human hippocampal formation is organized in a sequential manner as part of the telencephalon vesicle, from medial to lateral:

Dentate Gyrus → Cornu Ammonis → Subiculum → Entorhinal cortex

At this stage of development, there is a proliferation of neurons and glia in the neocortex. This tremendous expansion of tissue mass in the neocortex layer, along with the traction of neuronal projections, has been hypothesized to drive the folding of human cortex[168]. In the hippocampus, the folding occurs inwards and into *itself*, perhaps due to the fact that this cortex is at the *limbus* and there is no additional traction forces to form a gyrus (note fiber staining in Figure 1.7. lower left, which shows the difference between the white matter tract located underneath the parahippocampal gyrus vs. the hippocampus). In support of this hypothesis, comparative anatomy studies of the hippocampus in different species demonstrate that the degree of in folding of the hippocampal formation strongly correlates to the increase in neocortical cerebral hemispheres[169]. Inward folding takes place over the following 6-8 weeks until gestational age 18-21 weeks and occurs along the axis of the hippocampal sulcus that becomes progressively obliterated[170,171].

The hippocampal formation is a connectivity node, receiving and integrating multimodal afferents as well as sending efferents to multiple parts of the brain. Afferents from prefrontal cortices and sensory cortices connect to the entorhinal cortex (EC). The EC sends projections via its pyramidal neurons located on layer two and some of these projections go through (perforate) the lamina to make an excitatory (glutamatergic) synaptic contact with the granule cells in the granular layer of the dentate gyrus (DG). The granule cells of the DG project their mossy fibers

(axons) to CA3 where they make an excitatory (glutamatergic) synaptic contact with granule cells in the granular layer. CA3 pyramidal cells send an axonal projection to CA1 to make the third excitatory (glutamatergic) synaptic contact of this circuit, best known as the tri-synaptic excitatory pathway of the hippocampus (Figure 1.9). The EC also projects directly to CA3 and CA1, and it is hypothesized that the computational difference between the trisynaptic pathway and the direct pathways has a critical role in the multiple functions of the hippocampus. In order to accomplish its function, the hippocampus also relies on hippocampal interneurons, an extensive network of non-pyramidal cells that utilize GABA as their main neurotransmitter. Hippocampal interneurons can be distinguished by their morphology as well as molecular profile. There is a highly specific distribution of hippocampal interneurons based on their calcium-binding proteins (parvalbumin, carciretinin, calcineurin) and other proteins[167]. The hippocampal formation's main efferents are sent via connection to the subiculum and then fornix to the amygdala, nucleus accumbens, hypothalamus, thalamus, cingulate cortex and mammillary bodies as well as via connections in the EC and efferents to prefrontal cortices, mainly the medial, orbitofrontal and dorsolateral prefrontal cortices[172,173].

Functionally, the hippocampus is a key area in many cognitive processes, including verbal and non-verbal memory, and also plays a critical role in emotion regulation through its connections to the amygdala and the entire limbic system[172,174]. Using fMRI, the hippocampus has been subdivided into an anterior and a posterior portion with somewhat subspecialized functions, as the anterior pole has been more involved in emotion regulation and the posterior more devoted to cognitive processes[175].

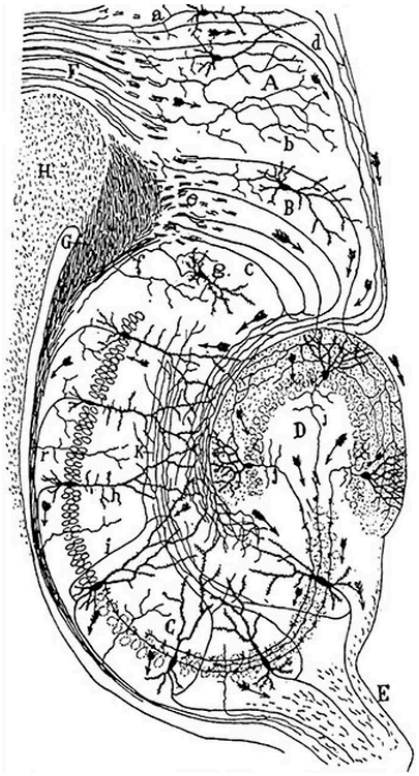

Figure 1.9 Hippocampus, histological structure and connections

Original drawing of the histological structure of the *cornu Ammonis* by Santiago Ramon y Cajal[j] [176]. A, occipital end ganglion; B, subiculum; C, cornu Ammonis; D, fascia dentate; E, fimbria; F, cingulum; G, cross spheno-ammoni cord; H, corpus callosum; a, penetrant axons into the cingulum; b, terminal cingular fibers in the occipital end; c, perforant spheno-ammoni fibers; d, perforant cingular fibers; e, plane of the superior spheno-ammoni fibers; g, subicular cells.

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[j] Figure 795 of the second part of the second volume. I have directly translated the text from the original even though there are terms that are no longer in use or have been revisited.

This model has been revisited and further sophisticated to incorporate more recent evidence showing there is a continuum of change or a gradient along the longitudinal axis rather than two distinct functional areas[177].

1.6.1.2 Neuropathological evidence of hippocampal abnormalities in schizophrenia

Evidence from neuropathological, cellular and molecular biology, and neuroimaging have shown that abnormalities in the hippocampal formation are very likely to be of central relevance to the etiopathogenesis of SCZ.

As discussed, there is clear evidence showing that SCZ is not grossly neurodegenerative as there is no overt demonstration of neuronal loss or reactive gliosis, both of which are cardinal neuropathological criteria to define a neurodegenerative process[154,178,179]. Rather, it is subtle neuropathological abnormalities that have been found in the number, density, size, shape, orientation and location of neurons in the hippocampal formation in SCZ. In healthy human post-mortem samples, hippocampus volume is estimated to be in the range of 3-5cm^3 after shrinkage correction[180]. Post-mortem studies in SCZ have consistently shown hippocampal formation bilaterally reduced in volume by 4% (albeit somewhat more pronounced in the left side[181]). This volume reduction has been robustly and consistently seen *in vivo* in structural MRI (sMRI) and is estimated to be around 5% decrease in a robust demonstration of convergence with post-mortem data. The majority of most post-mortem studies have reported a reduction in the total number of neurons[180] or in neuronal density[182–184], but some have reported reduction in number of subpopulations of neurons, particularly parvalbumin-positive hippocampal interneurons[185]. Observations of abnormal hippocampal neuronal morphology have been equivocal[184,186]. The most consistent finding involves abnormalities in the location of specific

neurons in the hippocampus, particularly the clustering and misplacing of pre-alpha neurons outside of layer II of the EC[130,187–190]. This finding, in addition to the disarray of pyramidal neurons, would support the hypothesis that abnormal migration of cells during development plays a role in the etiopathogenesis of SCZ and lends credence to the neurodevelopmental hypothesis of SCZ[191–193].

Post-mortem human brain studies have also revealed molecular abnormalities in the hippocampus. Molecular biology techniques have demonstrated abnormalities linked to both glutamatergic and GABA cell populations, and some evidence points to the possibility that these two systems might be affected differentially depending on the stage of the disorder, with older vs. younger patients preferentially showing glutamatergic abnormalities[194]. In addition, synaptic proteins, including synaptophysin, synapsin, SNAP-25 and complexins, have been shown to be decreased in the hippocampal formation in SCZ[195–197], implying a disruption in synaptic connectivity[198]. The vast majority of available evidence does not support an effect of neuroleptic exposure as an underlying cause of observed hippocampal deficits.

1.6.1.3 Neuroimaging abnormalities involving the hippocampus in schizophrenia

Abnormalities of the hippocampal formation are one of the most robust and consistent findings reported in the neuroimaging literature. The evidence encompasses structural, functional, and neurochemical abnormalities from an ample array of *in vivo* imaging techniques that include but are not limited to sMRI, functional MRI (fMRI), proton (^{1}H) magnetic resonance spectroscopy (^{1}H-MRS), diffusion tensor imaging (DTI), and positron emission tomography (PET). In this section, findings from functional and biochemical imaging will be reviewed.

Functional deficits and abnormalities of the hippocampal formation have been ascertained mainly with fMRI and PET. The literature is vast and converging evidence points on

the one hand to an increased level of metabolic activity and perfusion of the hippocampus that is correlated with the severity of psychotic symptoms, is particularly prominent during acute or subacute states, and normalizes with treatment response to antipsychotics[199–204]. Additionally, fMRI studies have associated abnormal patterns of activity involving the hippocampus with both cognitive impairment and specific psychotic symptoms[205–207].

Concordant neurochemical findings in the brain from MRS have been reported in SCZ. A meta-analysis by Steen and colleagues of ^{1}H-MRS studies in SCZ reported a ~5% reduction in N-acetylaspartate (NAA) bilaterally in the hippocampus as the most robust finding, in addition to reductions of NAA in prefrontal cortices[208]. More recent studies have also reported increased levels of glutamate/glutamine bilaterally in the hippocampus, as well as an association of glutamate/glutamine level with psychotic symptoms[209,210]. Some of these abnormalities may be present in unaffected relatives of SCZ probands[211,212].

1.6.1.4 Hippocampal formation structural abnormalities in schizophrenia sMRI

Reduced volume and abnormal shape of the hippocampus, and ventricular enlargement are the most consistent brain abnormalities found in SCZ. Bilateral volume reduction is seen in early stages of the disorder, in individuals at high risk of developing the disorder and in relatives of SCZ probands. In addition, some studies have reported mild progression of the volume loss in chronic SCZ[213,214] although this finding has not been universally replicated[215].

In sMRI studies, and similar to what has been reported in post-mortem human brain studies, hippocampal volume is estimated to be around ~4cm^3, and slightly bigger in the right side and in males. Two recent meta-analyses have provided robust indication of ~4% hippocampal volume reduction (i.e. ~160mm^3) in SCZ[216,217]. Whereas the study by van Erp et al. utilized prospective meta-analytic techniques, Haijma and colleagues used a more traditional

systematic review of the literature and applied meta-analytic statistics to analyze for effects. Van Erp and colleagues analyzed data from 15 independent cohorts of SCZ patients and controls recruited worldwide and applied prospective analyses of already collected data using standardized pre-processing, data capturing, and post-processing tools. Prospective meta-analysis utilizes summary statistics as opposed to mega-analyses that require the use of pooled individual data. Applying this methodology, they found individuals with SCZ had significantly (Bonferroni corrected) smaller hippocampus (mean difference from control mean: − 4.10%) and this was the largest SCZ vs. control effect size observed ($d = -0.46$, $p = 4.85 \times 10^{-14}$); even greater than the effect size for larger lateral ventricles [216]. Medication-naïve patient groups and younger age of onset were associated with smaller hippocampi. These findings are consistent with a deleterious effect of untreated psychosis on the hippocampal formation, and would suggest that early intervention to decrease the duration of untreated psychosis may have a beneficial effect in halting the progression of volume loss when treated[218].

Haijma and colleagues conducted a large, retrospective systematic review and meta-analysis of the literature on sMRI abnormalities in SCZ and included 117 studies. The analyses included a sample size of 8,327 medicated patients and 8,292 controls as well as 771 unmedicated patients and 939 controls (including 33 studies in antipsychotic-naïve patients). The large sample size and available subpopulations allowed the authors to investigate 100 brain structures as well as several moderator effects including duration of illness, antipsychotic dose, and sex[217]. With respect to the hippocampus, 68 studies were accessed with a total of 2,487 patients and 2,654 controls. The effect size for hippocampal volume was large and highly significant $d = -0.52$, 95% CI (− 0.60 to − 0.44), $p < 1 \times 10^{-9}$. This was the second largest subregional effect size after grey matter volume reduction of the superior temporal gyrus,

highlighting the temporal lobe as a key region of interest in SCZ. The hippocampal volume reduction effect size reported in by Haijma and colleagues is very similar to that found by Van Erp and colleagues[216]. Van Erp et al's subanalysis of medication-naïve patients comprised eight studies including a total of 194 patients and 251 controls (the vast majority of patients were first-episode SCZ patients). The volume reduction effect size was also large and highly significant $d = -0.43$, 95% CI (−0.63 to −0.24), $p = 7.6\times10^{-6}$. In this subsample, the largest effect size for volume reduction was the thalamus, but volume reduction in the hippocampus was again the second largest effect size. Meta-regression analyses of the hippocampus did not show any laterality, age of onset, medication, or sex effects.

Structural MRI investigation of the hippocampus has also provided fruitful insights utilizing shape analysis. Bilateral inward deformations, relatively more pronounced in the anterior portion of the hippocampus in first episode and chronic patients with SCZ, their unaffected relatives, as well as in individuals at risk have been reported[219–222]. Furthermore, medication effects did not account for these shape abnormalities[219–222]. The underlying mechanisms contributing to both hippocampal volume and shape deficits in SCZ seen from *in-vivo* imaging may be rooted in neuronal deficits observed in post-mortem samples. Arnold and colleagues described neuron size abnormalities were particularly located in subiculum, CA1, and layer II of the entorhinal cortex[184] in SCZ. Similarly, Matthew and colleagues found significant reductions of mean volume in CA1, CA2/3, CA4/DG, presubiculum, and subiculum of the hippocampal subfields in chronic SCZ patients compared to CTRL, although the most prominent differences were found in CA 2/3[223]. This finding was replicated by two separate research groups[224,225], and a third group expanded findings to a small sample of first-episode SCZ where they found that chronic and first-episode shared CA4/DG reductions, while only chronic SCZ

showed CA2/3 reductions[226]. A recent more comprehensive study by Ho and colleagues who analyzed two separate samples of patients with SCZ (recent onset SCZ and chronic SCZ), found that in early stages the volume reduction was mostly circumscribed to CA1, but in latter stages of the disorder the volume reductions expanded to all the other subfields. They estimated a progressive volume loss of ~2% per year and the progression of structural abnormalities with worsening of negative symptoms[227]. Hippocampal volume reductions have been quite consistently correlated with primarily negative symptoms[223,225,226], although one study found a correlation with only positive symptoms[228]. Decrease in hippocampal subfield volume has been associated with declarative memory impairment[223].

In summary, hippocampal volume reduction and shape abnormalities are robust and consistent findings. They occur bilaterally and are already present in early stages of the illness, and may slightly progress with the course of the illness. Medication effects do not account for these volumetric differences. In addition, sMRI studies in relatives of SCZ probands have detected slight hippocampal volume reductions, indicating that the volume loss is potentially linked to common genetic risks present in SCZ probands and their unaffected relatives[222,229–234].

1.7 Thesis dissertation chapters summaries and hypotheses

The thesis has undertaken the investigation of the pleiotropic effects of apolipoprotein varepsilon 4 on the SCZ syndrome using a translational strategy. Our findings demonstrate that APOE-ε4 influences several apparently unrelated phenotypes such as metabolism, signal transducing in a pathway involved in synaptic plasticity, hippocampal volume, and verbal memory performance. In addition, our finding of pleiotropic effects includes different stages across the lifespan as both a chronic SCZ sample, as well as a FEP sample was investigated. This represents an attempt to address the question of whether the pleiotropic effects of APOE-ε4 may

be antagonistic depending on the stage across the lifespan. These phenotypes investigated in the dissertation are translational as they address questions at different epistemological levels of emerging complexity, namely 1) Molecules → 2) Tissue/Organ → 3) System/Behavior (see Figure 5.2). Lastly, the experimental findings from the three parts lead to the emerging question as to whether APOE-ε4 may be associated with a particular syndromic presentation in SCZ, or whether APOE-ε4 may be a valid biomarker associated with a distinct nosological entity within the SCZ syndrome (Figure 1.3). Although this question is beyond the scope of this dissertation and not explicitly tested, it is an emerging hypothesis resulting from the set of findings, and it will be discussed in the section about future directions.

A translational pathway: molecules, brain structure, and behavioral outcomes

1) Molecular level

First, a human post-mortem sample of BA9 from patients with chronic SCZ and controls was used to address questions related to protein expression of apoE and related molecules in the LDLR signaling pathway, as well as genotypic regulation of APOE on these molecules. In addition, this sample also addressed questions related to the relationship of apoE to cholesterol in the human brain, as well as genotypic influence of APOE on cholesterol metabolism.

2) Tissue/Organ

Second, a sample of first-episode psychosis and healthy volunteers was used to address questions pertaining to effect of APOE genotype on brain morphometry, hippocampal volume, as well as the relationship of brain morphometry to behavioral measures including clinical outcomes and cognitive measures.

3) System/Behavior

Third, a sample of first-episode psychosis and healthy volunteers was used to address questions pertaining to the effect of APOE genotype on a behavioral domain: verbal memory performance.

Chapter 2: Quantification of Apolipoprotein E, cholesterol, reelin, apoER2, VLDLR, and methylation of reelin regulatory region in schizophrenia[k]

2.1 Introduction to chapter 2

The molecular and cellular mechanisms leading to SCZ are complex and multifaceted, but there is converging evidence pointing to alterations in synapses and myelin[235–237]. Cholesterol, apolipoprotein E (apoE), reelin and their common receptors apoER2 and very-low density lipoprotein receptor (VLDLR) comprise an excellent example of a complex molecular system with a potential role in the etiopathogenesis of SCZ.

2.1.1 Cholesterol, apoE, reelin, apoER2, and VLDLR multifunctional roles and relevance to synaptic function

Cholesterol is an essential molecular component of both synapses and myelin, and plays a multitude of functions in the brain. Structurally, it forms an integral part of cell membranes as well as a major component of myelin. Functionally it plays a critical role during brain

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[k] Versions of this chapter have been published:

A. **Vila-Rodriguez F**, Honer WG, Innis SM, Wellington CL, Beasley CL. ApoE and cholesterol in schizophrenia and bipolar disorder: comparison of grey and white matter and relation with *APOE* genotype. J Psychiatry Neurosci 2011 Jan; Vol. 36(1):47-55. PMID: 20964956.

B. Beasley CL, Honer WG, Ramos-Miguel A, **Vila-Rodriguez F**, Barr AM. Prefrontal fatty acid composition in schizophrenia and bipolar disorder: Association with reelin expression. Schizophr Res. 2017 Jun 2. pii: S0920-9964(17)30309-2. PMID: 28583708.

development, and as a key player for synapse homeostasis and function. Similarly to other aspects of CNS physiology, cholesterol homeostasis in the brain is privileged and autonomous from that of the rest of the human body as cholesterol is synthesized *in situ* by astrocytes and oligodendrocytes and is independent of that circulating in plasma. In the brain, essentially all cholesterol (>99.5%) is unesterified. The majority of cholesterol is believed to reside in two major pools: one in myelin sheaths, which represents approximately 70% of all brain cholesterol, and a second as part of cellular membranes and vesicles in all cellular types in the brain[61].

In addition, the lipid composition of neurons includes phospholipids either saturated- or polyunsaturated fatty acids (SFAs and PUFAs respectively) which primarily consist of arachidonic (AA) and docosahexaenoic acid (DHA). Similarly to cholesterol fatty acids are also critical in brain development[238], and the study of deficient level of FA has recently shown to potentially play a role in neuropsychiatric disorders and schizophrenia in particular[239]. Specifically, decreased levels of alpha linolenic acid and linoleic acid, also known as ω−3 and ω−6 fatty acids, have been shown in schizophrenia. Since this both fatty acids are essential (i.e. human organism does not synthetized them and the source is entirely through diet), clinical trials supplementing with ω−3 and ω−6 have been conducted in schizophrenia, although outcomes have been either negative or a small effect size[240–244].

ApoE is a 299aa, ~34 kDa, arginine rich, glycosylated, two-domain protein encoded by the apolipoprotein E gene (*APOE*), located on chromosome 19. In the human *APOE* gene, single nucleotide polymorphisms lead to the three main alleles, ε2, ε3, and ε4, which specify three protein isoforms, apoE2, apoE3 and apoE4, that differ by a cysteine-arginine substitution at residues 112 and 158[35,36,245]. Synthesized by astrocytes and activated microglia, apoE is the

major apolipoprotein in the brain, where it forms HDL-like particles that distribute cholesterol among neurons and their supporting cells[49].

ApoE and cholesterol both play an essential role in synaptogenesis, neurite outgrowth, and membrane repair and maintenance, including that of the myelin sheath[59,246–248]. When co-cultured with glial cells, neurons develop more synapses and those synapses are more efficient compared to non- co-cultured neurons[249]. Both apoE and cholesterol were determined to be necessary factors implicated in this process[59]. Cholesterol and apoE also play a role in the regulation of neurite outgrowth[57], where there is an allele specific efficiency with apoE3 increasing neurite outgrowth, but apoE4 having the opposite effect [247]. Cholesterol is continuously metabolized in the brain, whereby unesterified cholesterol is either recycled back to myelin or plasma membranes, or hydroxylated and secreted to plasma; apoE once again is the main transporter involved in these processes[60].

The **low-density lipoprotein receptor (LDLR)** family is a highly evolutionary conserved group of single transmembrane proteins that have a very diverse array of functions[250]. Figure 2.1 displays a schematic representation of the seven members of this family of proteins and show the remarkable structural similarities they share: i) single transmembrane domain; ii) ligand binding type domain repeats; iii) epidermal growth factor (EGF) precursor homology domain; iv) transmembrane domain, and v) NPxY motif in the N-terminal region which is an interaction site for intracellular adaptor proteins that transduce signal to downstream intracellular signaling pathways.

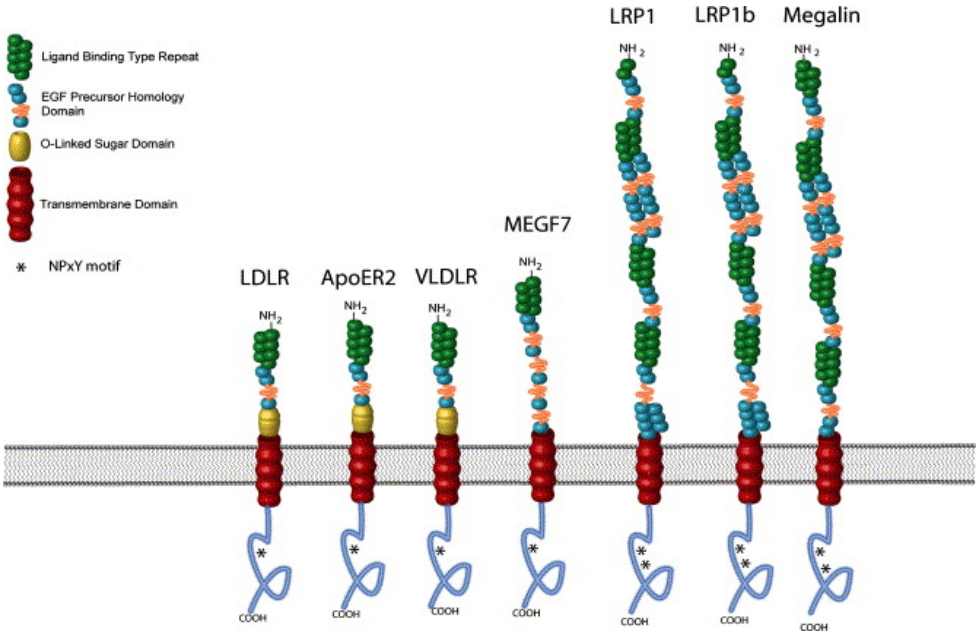

Figure 2.1 Low density lipoprotein receptor family (LDLR)

Low density lipoprotein receptor family (LDLR) is represented in this figure showing the seven receptors of this highly conserved family of proteins and the different common domains and motifs in the receptor family. Figure from[250] with permission.

ApoE is a high affinity ligand to **all** the LDLR family members as well as other lipoproteins such as apoB or apoJ in consistency with the role of LDLR in lipid metabolism. LDLR have remarkably evolved to play a role in an array of very diverse functions not related to lipid metabolism. Evidence for this functionality beyond lipid metabolism is the growing number ligands associated with other functions; amyloid precursor protein, tissue plasminogen, a2-macroglobuilin, or reelin are just four examples of a growing list of LDLR ligands associated

with other functions not related to lipid metabolism, most importantly in the brain where LDLR are associated with synaptic plasticity, learning and memory[251].

Reelin is a large 3461aa, glycosylated, 410 kDa extracellular matrix protein codified by the reelin (*RELN*) gene located on chromosome 7q22[252,253]. Reelin plays a critical role during brain development as a key player in guiding neuronal migration and contributing to the normal folding of the cerebral and cerebellar cortex. Absence of reelin during development is associated with severe abnormalities in neuronal migration. In adulthood, reelin is produced by GABAergic interneurons and is associated with the postsynaptic density, dendritic spines and axons throughout the cortex as well as hippocampus, where it plays a role in synaptic plasticity, memory and learning. Reelin selectively binds to only two of the receptors within the LDLR family: apoER2 and VLDRL[254,255]. One mechanism to transduce signals into the cell is by interacting with apoE and clustering apoER2 and VLDRL with apoE. The downstream effects involve Disabled-1 (Dab1), which mediates intracellular signaling cascades and modulates N-methyl D-aspartate receptor (NMDAR) activity[250]. The interaction of apoE and reelin in clustering both receptors and transducing intracellular signals is shown in Figure 2.2. Animal models deficient in both apoER2 and VLDRL or Dab1 are associated with a similar phenotype as reelin mutant animals[256], which display cognitive deficits and impaired sensorimotor gating[257–259].

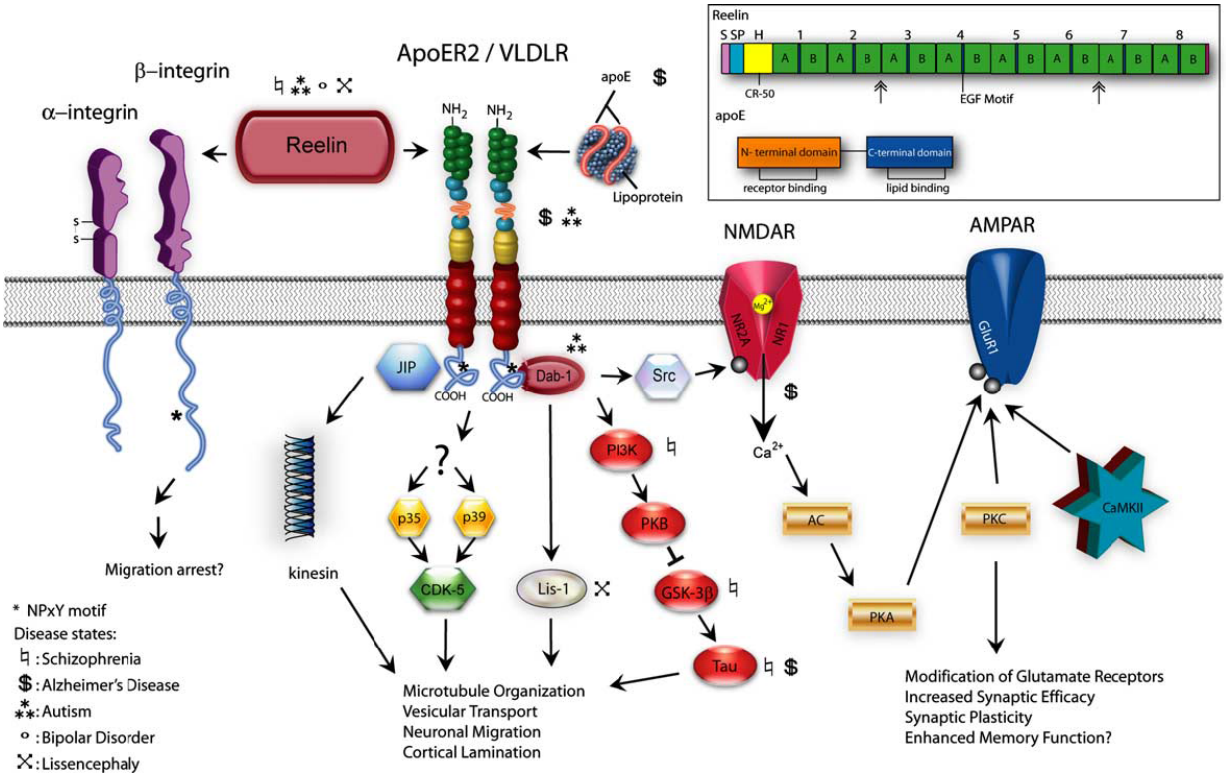

Figure 2.2 ApoE and reelin interaction with LDLR and impact on synaptic plasticity

This figure schematically shows role of apoER2, VLDLR, reelin and apoE in transducing endocytosis-mediated signals into the cell and the downstream effects on several signaling pathways through adapter proteins. Of particular relevance in adulthood is the effect on NMDAR and alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR). The figure also shows sites along the pathway that have been implicated in pathological states. From Qiu et al[250] with permission.

2.1.2 Cholesterol, apoE, reelin, apoER2, and VLDLR in schizophrenia

While altered lipid levels have been identified in the periphery in SCZ[260–262], to date there have been few studies in brain tissue. One study investigating a heterogeneous diagnostic sample, including affective as well as psychotic disorders, found that cholesterol levels were lower in Brodmann areas (BA) 11 and 47 in individuals who had completed suicide by violent means[263]. Dean and colleagues found increased apoE in BA 9 and 46, with no difference in BA10 or the striatum in SCZ, whereas apoE was lower in BA 10[126,127]. Finally, a recent meta-analysis reported that the *APOE*-ε4 polymorphism was significantly associated with SCZ[134], although a more recent systematic review and meta-analysis shows a discrepant result[133].

Overall, few studies have assessed cholesterol and apoE abundance in postmortem brain in the major psychiatric disorders. Despite differences in lipid composition between grey and white matter regions, neither apoE nor cholesterol levels have yet been quantified in white matter in SCZ. The relationships between *APOE* genotype, apoE protein levels and cholesterol content have not yet been fully resolved. While studies in AD patients[264] and transgenic mice[265] have reported a genotype-dependent decrease in apoE levels (ε2/2 > ε3/3 > ε4/4) this finding has not been thoroughly replicated in human samples[266]. Studies in transgenic humanized apoE mouse models show that homozygous *APOE*-ε4 animals have decreased levels of apoE4 in brain, plasma, and CSF in spite of having greater mRNA levels than *APOE*-ε2 and *APOE*-ε3 homozygous animals. In *APOE*-ε3/ε4 heterozygous animals, apoE4 represents a 30% of the total apoE, and the absolute amount of apoE3 per allele is similar to that of APOE-ε3/ε3 homozygous mice. This suggests that the reduced levels of total apoE in *APOE*-ε3/ε4 heterozygous mice are caused by a reduction of apoE4 protein levels. In the same in vitro experiments, secreted cholesterol levels were lower in primary cultured astrocytes from *APOE*-ε4/ε4 homozygous

animals. In addition, in vitro experiments showed an enhanced degradation and reduced half-life of newly synthesized apoE4 compared with apoE3[265].

Converging evidence has demonstrated that abnormalities in either or several of the molecules involved in the signaling pathway described in Figure 2.2 may lead to neuropathological changes, abnormal cognitive processes, and psychosis-like behavior in animal models[257–259]. One prevailing model of SCZ, is of a neurodevelopmental origin[267]. One of the hypothesized neurodevelopmental mechanisms involves abnormalities in the migration of neurons using radial glia during development. Loss of reelin, one the key players in this process, leads to abnormalities in the lamination of cerebral and cerebellar cortex as well as hippocampus[254,256]. Barr and colleagues showed that in a rodent model of SCZ loss of either of the reelin receptors (i.e. apoER2 or VLDLR) both lead to similar impaired sensorimotor gating and neurocognitive deficits[257,258]. Converging with animal models, evidence in humans showed that reelin expression was decreased in SCZ patients[268–270], as well as showing that one of the potential factors mediating this decreased expression could be the finding of increased methylation of the promoter region of the *RELN* gene in SCZ patients[271,272]. This demonstrated that an epigenetic mechanism such as hypermethylation of the promoter region of the RELN gene could explain the decreased expression of reelin.

2.1.3 Aims and hypotheses

Aim 1. To investigate the effect of *APOE* on brain cholesterol metabolism and the LDLR signaling pathway involved in synaptic plasticity through quantification of molecules involved in brain cholesterol metabolism, and LDLR signaling transducing pathway involved in synaptic plasticity.

Aim 2. To investigate the effect of *APOE* on brain cholesterol metabolism, and the LDLR signaling pathway in SCZ.

Hypothesis 1.1. *APOE* genotype will be associated with apoE and cholesterol levels.

Hypothesis 1.2. *APOE* genotype will be associated with the expression of reelin mRNA and the methylation of its promoter region, apoER2, and VLDLR mRNA.

Hypothesis 2.1. SCZ will be associated with abnormal levels of apoE and cholesterol.

Hypothesis 2.2. SCZ will be associated with abnormal expression of reelin mRNA and the methylation of its promoter region, apoER2, and VLDLR mRNA.

2.2 Methods and materials

2.2.1 Brain tissue

Frozen samples of the dorsolateral prefrontal region (BA9), were obtained from the Stanley Foundation Neuropathology Consortium, MD, USA. Tissue was available from one hemisphere of each brain, with approximately equal numbers sampled in a random manner for each side, and was carefully dissected out into grey matter and adjacent white matter. The sample consisted of 70 subjects (35 controls with no known psychiatric or neurological disorder and 35 SCZ). Diagnoses were made according to Diagnostic and Statistical Manual of Mental Disorders IV edition criteria[273].

Detailed demographic postmortem, and clinical information is reported in Table 2.1. All brains underwent clinical neuropathological examination and none demonstrated evidence of neurodegenerative changes or other pathological lesions. Investigators were blind to both diagnosis and genotype when measuring cholesterol and apoE levels. None of the subjects from the control group died of suicide, and most died of acute illness such as myocardial infarction, pneumonia, or trauma. Detailed descriptions of causes of death are presented in Table 2.2.

2.2.2 Metabolomics: free cholesterol quantification using high-performance liquid chromatography (HPLC)

Cholesterol was separated from other lipids and quantified using a Waters Alliance 2695 HPLC equipped with an autosampler and evaporative light scattering mass detector (ESLD) as previously described[274]. Briefly, tissue was homogenized in 15 volumes of ice-cold tris-buffered saline (TBS) and protein quantified using a Lowry-based method (DC assay, BioRad). Total lipids were extracted from grey and white matter (wet weight, 13.33 and 6.67 mg, respectively) using a modification of the Folch method[275]. Sample homogenate was made up to a total of 2.5 ml with NaCl/EDTA in water (9 g/l/1.14 g/l), 3 ml methanol was added and the sample vortexed, then 6 ml chloroform was added, the sample vortexed, centrifuged to enable separation of the organic and inorganic phases. The organic phase was then recovered and transferred to a clean tube. The remaining inorganic phase was extracted 2 times to ensure complete recovery of all lipids, with organic phases combined and the solvent evaporated under nitrogen, then resuspended in 50 ml of a hexane/acetone/methanol/chloroform, 1/1/6/4 by volume containing 75 μg of betulin as the internal standard

	Group	
	CTRL (n = 35)	SCZ (n = 35)
Demographic		
Age at death in years, mean (SD)	44.2 (7.6)	42.6 (8.5)
Sex (male/female)	26/9	26/9
Ethnicity (C:H)	35:0	34:1
Brain postmortem characteristics		
Postmortem interval (hours), mean(SD)	29.37(12.87)	31.4 (15.54)
Brain pH, mean (SD)	6.61 (0.26)	6.47 (0.24)*
Storage (days), mean (SD)	2815 (538)	3026 (704)
Brain Hemisphere (right:left)	16:19	17:18
Clinical		
Cause of death	33a: 2c	21a:7b:7c
Age of Onset in years, mean (SD)	n/a	21.29 (6.07)
Duration of disorder in years (SD)	n/a	21.29 (10.15)
Antipsychotic dose in mg; median, minimum, maximum	n/a	48000, 50-400000
Alcohol History (none/mild:moderate:heavy)	30:3:2	17:6:12**
Drug History (none/mild:moderate:heavy)	34:1:0	18:6:9 (2 Missing)**
Death by suicide (no:yes)	35:0	28:7

CTRL = Control; SCZ = Schizophrenia; Ethnicity is categorized as follows: C = Caucasian; AM = African-American; H = Hispanic; NA = Native American. Cause of death is categorized under the following headings: a) Cardiopulmonary, b) Suicide, c) other. Statistical values: *p < 0.05; **p < 0.01;

Table 2.1 Demographic and postmortem characteristics of the sample

Case	Group	Cause of Death	Suicide	Case	Group	Cause of Death	Suici
1	Ctrl	Cardiac	No	1	SCZ	SUIC: J	Yes
2	Ctrl	Cardiac	No	2	SCZ	Cardiac	No
3	Ctrl	Cardiac	No	3	SCZ	Cardiac	No
4	Ctrl	Cardiac	No	4	SCZ	SUIC: OD	Yes
5	Ctrl	Cardiac	No	5	SCZ	SUIC: J	Yes
6	Ctrl	Cardiac	No	6	SCZ	Cardiac	No
7	Ctrl	Cardiac	No	7	SCZ	SUIC:HNG	Yes
8	Ctrl	Cardiac	No	8	SCZ	Cardiac	No
9	Ctrl	Cardiac	No	9	SCZ	SUIC: OD	No
10	Ctrl	Myocarditis	No	10	SCZ	SUIC:HNG	Yes
11	Ctrl	Cardiac	No	11	SCZ	Cardiac	No
12	Ctrl	Cardiac	No	12	SCZ	SUIC: J	Yes
13	Ctrl	Cardiac	No	13	SCZ	Cardiac	No
14	Ctrl	Cardiac	No	14	SCZ	Pneumonia	No
15	Ctrl	Cardiac	No	15	SCZ	Cardiac	No
16	Ctrl	Cardiac	No	16	SCZ	Cardiac	No
17	Ctrl	Cancer	No	17	SCZ	Cardiac	No
18	Ctrl	Cardiac	No	18	SCZ	Cardiac	No
19	Ctrl	Cardiac	No	19	SCZ	Cardiac	No
20	Ctrl	Cardiac	No	20	SCZ	MVA	No
21	Ctrl	Cardiac	No	21	SCZ	Cardiac	No
22	Ctrl	Cardiac	No	22	SCZ	Cardiac	No
23	Ctrl	Asthma	No	23	SCZ	Cirrhosis	No
24	Ctrl	Cardiac	No	24	SCZ	Cardiac	No
25	Ctrl	Cardiac	No	25	SCZ	Pneumonia	No
26	Ctrl	Cardiac	No	26	SCZ	Pulmonary Emb	No
27	Ctrl	Cardiac	No	27	SCZ	SUIC:HNG	Yes
28	Ctrl	Cardiac	No	28	SCZ	Cardiac	No
29	Ctrl	Cardiac	No	29	SCZ	Pneumonia	No
30	Ctrl	Cardiac	No	30	SCZ	Pneumonia	No
31	Ctrl	Pulmonary Emb	No	31	SCZ	SUIC: OD	Yes
32	Ctrl	Cardiac	No	32	SCZ	Pancreatitis	No
33	Ctrl	Cardiac	No	33	SCZ	SUIC: OD	Yes
34	Ctrl	Cardiac	No	34	SCZ	Cardiac	No
35	Ctrl	Cardiac	No	35	SCZ	Pneumonia	No

Ctrl = Control; SCZ = Schizophrenia; SUIC = Suicide (OD = overdose; J = Jump; HNG = hanging

Table 2.2 Cause of death detailed case summaries

Lipids classes including unesterified cholesterol were separated using a YMC diol 4.6 mm X 250 mm column at 35°C with a quaternary gradient of A: hexane, B: methanol, C: 1.7% triethylamine in acetone and D: 0.5% acetic acid in isopropanol. The quantity of cholesterol was determined from the area ratio of cholesterol to betulin, which was constant in all sample injections. The detector response is linear for unesterified cholesterol over the range of to 0.1 to 2.4g/l with a relative response of cholesterol to betulin of 1.24:1. Data were expressed as μg cholesterol per mg protein.

2.2.3 Proteomic analyses: apoE quantification with enzyme-linked immunosorbent assay (ELISA) and western blot

The ELISA method followed the general methodology for relative quantification of proteins in their native conformation developed in the lab and used for the last 30 years. This method has been used and reported in the literature[276–279], and conceptually can be regarded as a variation of the well-known methods described by Bradford in 1976[280]. Briefly, quantity of tissue to analyze is standardized to a given concentration of protein. Each sample is serially diluted in duplicates and each ELISA plate contains a duplicate control serial dilution. The concentration of primary antibody is added to samples, but not to the control dilution. Subsequently, secondary antibody is added to both samples and the control to account for unspecific protein binding. Once the colorimetric reaction occurs optical density is read. Thus the optical density (OD) read for a sample decreases as the amount of protein of interest decreases yielding a curve of values the majority of them in the linear range. The last step consists of selecting an OD that falls in the linear range for all the samples. The concentration of protein at this OD in arbitrary units is used as protein concentration to compare across samples.

This method differs from the ELISA methods that use a protein standard. One of the main limitations of the assay is that the quantification of protein is not in absolute values, and therefore quantification of sensitivity measures such as lower limit of quantification (LLOQ), lower limit of detection (LLD), or upper limit of quantification is not possible. These measures may be critical in situations where the protein of interest is not abundant and a very sensitive assay is needed to establish the presence or absence of the protein. In situations, where the protein of interest is very abundant, however, quantifying the sensitivity of the assay may be less critical. In addition, the Bradford method allows to infer relative quantification of proteins in their native conformation in the tissue which may be relevant for proteins that are in present in tissue in different quaternary structures or in modified states (e.g. forming dimers, tetramers, sialylation). In this regard, recent work from the lab where the assay was developed, compared the quantification of proteins between the modified Bradford method and quantitative mass spectrometry and demonstrated that modified Bradford method produced accurate relative quantification of proteins[278].

An optimized ELISA assay was developed to reliably measure the three main apoE isoforms in human postmortem tissue. This process involved the systematic testing and comparison of parameters in the ELISA method including i) type of plate used, ii) the use of detergent, iii) type of incubation, iv) incubation temperature and time, v) protein concentration, vi) testing of several primary antibodies, vii) concentration of primary antibody, viii) concentration of secondary antibody and ix) type of substrate. The experiments informed the choice of the following parameters for the final assay: i) use of Nunc MaxiSorb plate (Thermo Fisher, Waltham, MA, USA), ii) no use of detergent, iii) wet incubation of sample, iv) overnight incubation at 4 ^{0}C, v) protein concentration of 240ug/ml, vi) use of a mouse monoclonal IgG1

anti human apoE as the primary antibody (ATCC catalogue number CRL-2255, epitope in amino acids 126 through 191, Manassas, VA, USA), vii) concentration of primary antibody 1:10, viii) concentration of secondary antibody 1:1000, and ix) use of 3,3',5,5'-tetramentylbenzidine as the substrate (TMB - KPL, Gaithersburg MD, USA). The optimization flow and decisions made based on systematic experiments with human postmortem brain tissue are detailed in Figure 2.3.

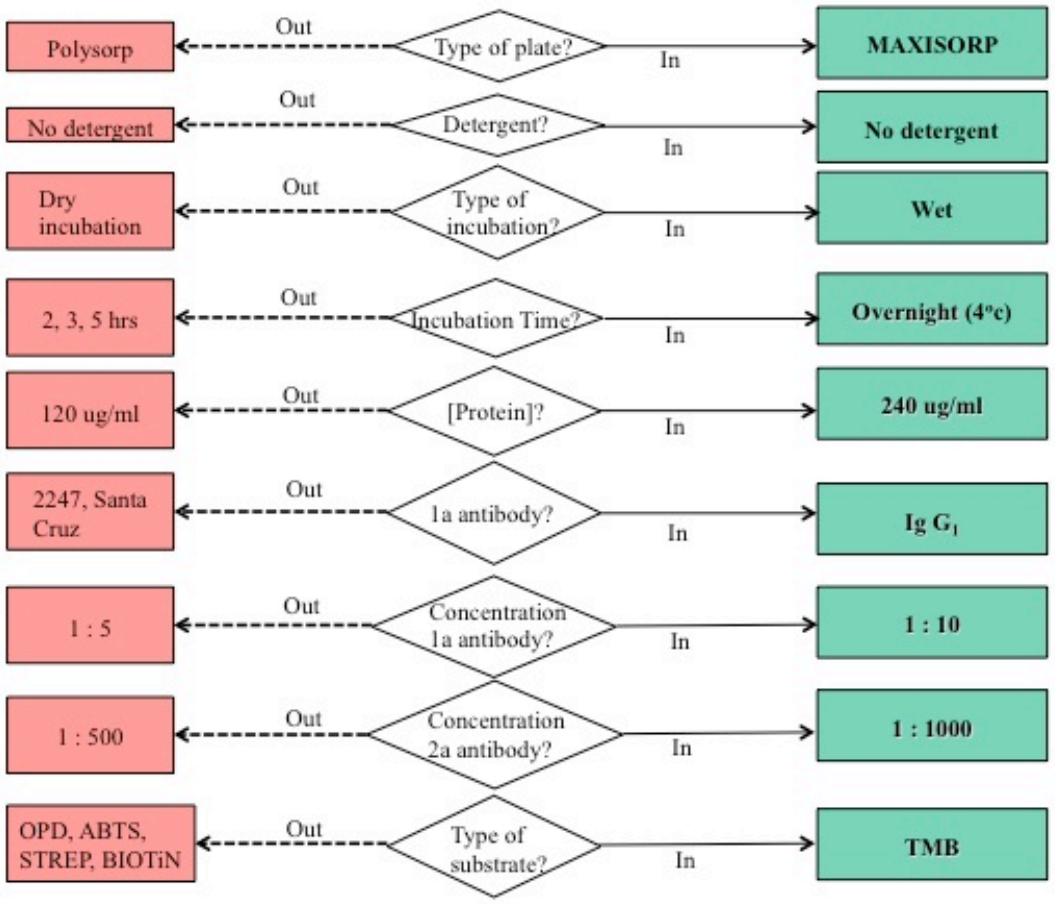

Figure 2.3 ELISA optimization flow chart

Systematic testing of assay was conducted to decide final assay procedure as summarized in this figure.

The optimized assay was used to conduct ELISA experiments. Tissue homogenates were diluted to 240-μg protein/ml in TBS. Duplicate samples were then serially diluted over a 128-fold range and incubated overnight at 4 ^{0}C in 384 well ELISA plates. Non-specific binding was blocked using TBS containing 5% milk. Plates were then incubated with primary antibody overnight at 4 ^{0}C. Each plate also contained negative control wells in which tissue culture-conditioned media was substituted for the primary antibody. The plates were further incubated with peroxidase-conjugated secondary antibody targeted to IgG_1 at 1:1000 concentration (IgG goat anti mouse polyclonal Ab, Jackson Immunoresearch Laboratories catalogue number 115-585-062, West Grove, PA, USA). Finally, TMB was added and after 30 minutes the reaction was stopped with 1M phosphoric acid and the optical density determined at 450nm.

The optical density of each well was plotted against the protein concentration and only the linear portion of the curve assessed for each sample. The assay was linear over an average 22 and 14-fold range for grey and white matter respectively. Samples were run twice, on different days, and mean values used for analyses. Between-run correlations were greater than 0.90. A serial dilution of a reference brain sample was run on each plate in order to compute a between-plate coefficient of variation. This coefficient of variation was calculated to be 5.1% and 5.7%. To compare immunoreactivity between samples and regions, the amount of protein required to give an optical density reading of 0.4 was used for both grey and white matter. Two cases were excluded from the white matter analysis, and one from the grey matter analysis, as an optical density reading of 0.4 did not fall within the linear range. The ELISA data values (total protein at a fixed optical density) are inversely related to the amount of target antigen present in a sample. For graphing purposes only, we employed a simple algebraic transformation to plot the data in

the intuitively simpler fashion where greater values represent greater amounts of the target antigen. This results in no distortion of the distribution of values.

2.2.4 Western blot analysis

Western blot experiments were conducted to confirm the specificity of the antibody. Briefly, 20 μg of brain homogenate were separated on a 10% SDS polyacrylamide gel. Following transfer to PVDF membrane (BioRad, Hercules, CA, USA), the blot was incubated with primary antibody 2255. The blot was further incubated with peroxidase-conjugated secondary antibody goat anti mouse 1:5000, for one hour. ECL reagent was added, and blots imaged using a LAS-3000 imager (Fujifilm, Minato, Tokyo, Japan). Using this antibody we were able to detect a doublet at the expected molecular weight of approximately 36 kDa (Figure 2.4), as well as a heavier 80kDa band. Doublets at around 36kDa have been described previously and are thought to result from apoE sialylation[49,281,282]. In addition, apoE may be observed as both a ~36-kDa monomer and an ~80-kDa dimer due to the presence of a cysteine at residue 112 in human apoE3[282,283] .

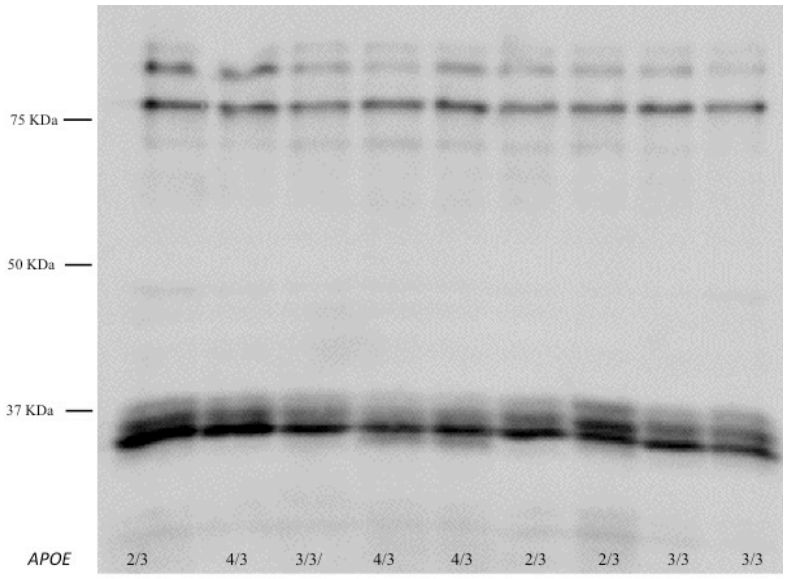

Figure 2.4 Western blot of apoE from human dorsolateral prefrontal cortex homogenate

Human dorsolateral prefrontal cortex (BA9) homogenates for all genotypes were used to identify apoE. Total protein (20μg) was diluted in reducing Laemmli buffer and run on a 10% SDS polyacrylamide gel. A ≈ 36-kDa duplet band, in addition to a ≈ 80-kDa band can be observed. Duplets are caused by different degree of apoE sialylation as previously described[49,282], while apoE complexes not dissociated under SDS process have been previously described at higher MW[282,283].

2.2.5 Reelin, apoER2 and VLDLR mRNA

Reelin, apoER2, and VLDLR mRNA, were determined in all samples. The RNA quality of each brain sample was assessed by the Stanley Foundation Consortium by measuring glyceraldehyde or actin mRNA and then grading the yield: A (excellent), B (good), C (fair), D (poor), and F (very poor); only samples graded A to C were used. All specimens are assessed for brain pH as studies have shown pH to be more associated with mRNA quality than PMI. A study

carried out on 89 specimens from the Stanley Foundation brain collection to ascertain the relationship of pH and PMI to mRNA was made and it was found that the quality of mRNA deteriorated with conditions of agonal hypoxia (e.g. carbon monoxide poisoning) and that pH seemed to be a reasonably good measure of mRNA quality (i.e. the higher the pH, the better the mRNA)[284,285].

The methods for reelin mRNA and methylation, as well as apoER2, and VLDLR mRNA levels are described in detail by Tamura and colleagues[286] (reelin methylation and reelin mRNA data) and Suzuki and colleagues[287] (apoER2 and VLDLR).

Briefly, the levels of methylation of the promoter region of was conducted by digesting genomic DNA with EcoRI and BssHII (methylation-sensitive restriction enzyme) at 37.1 C overnight, collected by ethanol precipitation, and dissolved in 50 ml of TE. Digested DNA (60 ng/test) was used as a template and was examined by means of real-time PCR using the following PCR primer sets: For amplification of the control region of RELN; RC-F1, 50-GAACAGTCCGGCGAAGAGAG-30 RC-R1, 50-CAGAGCCTCATCTGTAGAGGATTT-30 For the test region of RELN, we carried out real-time PCR with a RELN probe that we designed: RC-F3, 50-CGGCGTCTCCAAAACTGAATGA-30 RC-R3, 50-GTGGGGTTGCCCGCAATATGCAG-30 RELN probe, 50-(FAM)-CTAGCGCTGTTGCTGGGGGCGACGCTG-(TAMRA)-30. Real-time PCR was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR GREEN PCR Master Mix (for control region) or TaqMan Universal PCR Master Mix (for test region) according to the manufacturers' instructions and was repeated at least three times. The measurements of mRNA reelin were obtained by means of reverse transcription (RT)-(real-time) PCR with TaqMan Gene Expression Assays (Applied Biosystems)

using TaqMan probes specific for RELN (assay ID: Hs00192449_m1) and S18 rRNA (assay ID: Hs99999901_s1) as a control. The assay RT-PCR) was carried out using the ABI PRISM 7000 or 7300 sequence detection system (Applied Biosystems) with TaqMan One Step RT-PCR Master Mix Reagents kit (Applied Biosystems) according to the manufacturer's instructions and was repeated at least three times. mRNA levels for apoER2 and VLDLR were measured using RT-PCR where RNA preparations were resuspended in diethylpyrocarbonatetreated water and quantified by analyzing absorbance at 260 nm. Complementary DNA was prepared by incubating DNase-treated total RNA (1.0 μg) with M-MLV reverse transcriptase (Invitrogen, CA, USA) in the presence of random primers. RT-PCR analysis was performed for 55 cycles (95 °C for 30 sec, 60 °C for 45 sec, 72 °C for 30 sec) using ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, CA). TaqMan probes and primers were constructed according to known sequences for VLDLR (Genbank NM_003383) and ApoER2 (Genbank D50678) as follows: VLDLR, forward 5′-CTGCAGGGACTGGAGTGATGAG-3′, reverse 5′-GCAGATTCCTGGATTTTGGCA-3′, probe 5′-CGAGTGTGACTGTGCAGCTGGGTTTGA-3′); ApoER2, forward 5′-TGTGAGTGCTACCCTGGCTACGA-3′, reverse 5′-GCCTTGTCCATGTAGGCGCTATAG-3′, probe 5′-TCACCAACCGGTACGAGGT-GCGGAGG-3′.

2.2.6 *APOE* genotyping: Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP)

APOE genotyping was performed using PCR-RFLP, as described previously[288] but with slight modifications. Genomic DNA was isolated from 15-25 mg of brain tissue using a DNA purification kit (DNeasy blood and tissue kit, Qiagen). A 318 bp fragment from the *APOE* gene was PCR-amplified in 50 μl containing 10 μl (0.1–0.4 ng) purified genomic DNA, 1x Qiagen

PCR Buffer, 0.35 μM each primer, 200 μM each dNTP, 2x Q-Solution, and 1.5 U Qiagen Taq DNA Polymerase. Two primers were used in the amplification: upstream primer E2mut (5' ACT GAC CCC GGT GGC GGA GGA GAC GCG TGC) and downstream primer E3 (5' TGT TCC ACC AGG GGC CCC AGG CGC TCG CGG). Primer E2mut differs from the genomic sequence at one position, which creates an additional AflIII recognition site in the amplified fragment. Reaction mixtures were incubated at 94°C for 3 min, subjected to 40 cycles of amplification (95°C, 20 sec; 65°C, 40 sec; 72°C, 45 sec), and incubated at 72°C for 7 min. Restriction digests containing 15 μl amplification reaction (2.1 μl H2O, 0.15 μl BSA, and either 2 μl Qiagen buffer #3 and 0.75 U Afl III or 2 μl Qiagen buffer #4 and 0.125 U Hae II) were incubated at 37°C overnight. The digested product was run on 4% agarose gels, stained with ethidium bromide and visualized under UV light. Genotype was determined by comparison with standards run on the same gel.

2.2.7 Statistics

Shapiro-Wilks tests were used to determine if apoE and cholesterol measures conformed to a normal distribution. As data were not normally distributed, variables were transformed using a two-step approach consisting on first transforming it to a percentile rank and then application of an inverse-normal function[289]. After transformation, all variables were normally distributed. The results of the transformation are demonstrated with apoE levels in grey matter in Figure 2.5.

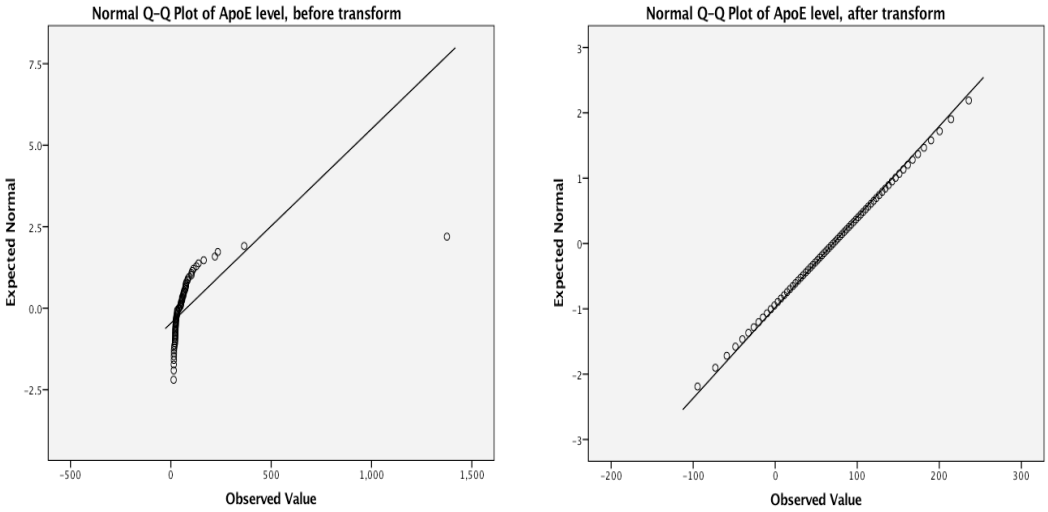

Figure 2.5 Q-Q plots of apoE levels before and after transformation

The normal Q-Q plots for the variable apoE level in grey matter are shown, before and after the transformation of non-normally distributed variables using the procedure described by Templeton[289].

The procedure described by Templeton provides a very robust method to normalize variables, and hence utilize parametric methods with the transformed variables. I conducted analyses using non-parametric methods (Spearman's rank; ρ) on the original variables (i.e. not normally distributed) for apoE, cholesterol levels, and age as well as with parametric methods for the transformed variables (Pearson's correlation; **r**). Results are summarized in table 2.3 and show that both analyses yield comparable results.

	Age	ApoE	Cholesterol
Age		**r** = .12; p = 0.33	**r** = -.07; p = 0.57
ApoE	**ρ** = .13; p = .27		**r** = .44; p < .001
Cholesterol	**ρ** = -.05; p = .67	**ρ** = .51; p < .001	

Table 2.3 Comparison of results using Spearman's ρ on original non-normally distributed variables or Pearson's correlation r on transformed variables

The directionality of association between variables, strength of association or the statistical significance is comparable with both methods applied to the corresponding variables.

Differences in age, postmortem interval (PMI), storage time, and brain pH between groups were assessed using a one-way analysis of variance (ANOVA), with significance level alpha = 0.05.

Pearson correlations were performed in order to assess the influence of age, PMI, storage time, and brain pH on apoE levels, cholesterol levels, methylation, and mRNA levels. When variables were statistically significantly correlated, they were included in the ANCOVA models. If statistical significance for the covariate in the model was below 0.1 they remained in the model and otherwise were removed from the final model.

In addition, exploratory analyses using one-way ANOVA were conducted to examine whether alcohol use (none or social versus past or present moderate or heavy use), illicit drug use

(none or social versus past or present moderate or heavy use), or the effect of death by suicide was associated with apoE, cholesterol, or mRNA levels. Pearson correlation was computed between lifetime dose of antipsychotics and apoE, cholesterol, or mRNA levels to explore relationships between these variables. Univariate analyses of variance (ANOVA) or covariance (ANCOVA) were conducted to investigate the effect of the psychiatric condition or *APOE* genotype on apoE, cholesterol, and mRNA levels. Independent samples t-test were conducted to test differences in apoE and cholesterol levels between grey and white matter. Pearson correlation was conducted between apoE and cholesterol and grey matter to explore the relationship of these two variables. Statistical significance was set at $\alpha = 0.05$ unless otherwise specified. All statistical analyses were computed using SPSS 22.0.

2.3 Results

2.3.1 Demographic, clinical, and postmortem variables

The potential confounding postmortem variables brain pH, postmortem interval (PMI), and time stored at -70^{o}C were examined. Postmortem interval did not correlate with apoE, free cholesterol, or mRNA levels. Brain pH was correlated only to reelin methylation ($r = -.26$; $p = .03$) and reelin mRNA levels ($r = .24$; $p = .05$). Storage time was correlated with levels of apoE in grey matter ($r = -.42$; $p < .001$), and reelin mRNA ($r = .30$; $p = .01$). Age was weakly correlated to ApoER2 mRNA ($r = .26$; $p = .04$).

Groups did not differ in age, sex, or ethnicity. Brain pH was higher in controls (CTRL) compared to the SCZ sample ($t(68) = -2.1$; $p = .036$; see table 2.1). Both groups had similar postmortem interval (PMI), storage time, and brain hemisphere. Neither apoE nor cholesterol levels differed as a function of sex or brain hemisphere. The SCZ group had a greater proportion of individuals with alcohol use and drug use history (see table 2.1.). Lifetime dose of

antipsychotics did not correlate with apoE or cholesterol levels, reelin, ApoER2, or VLDLR mRNA levels.

2.3.2 ApoE, cholesterol, reelin methylation, reelin, ApoER2, and VLDLR mRNA levels in schizophrenia

ApoE and cholesterol levels as well as reelin, ApoER2, and VLDLR mRNA levels for both groups in grey matter are presented in table 2.4.

	Schizophrenia	Control
ApoE GM; mean (SD)	58.9 (65.0)	82.1 (77.5)
Cholesterol GM; mean (SD)	148.4 (72.6)	161.3 (68.0)
Reelin meth; mean (SD)	0.0303 (0.034)	0.0119 (0.028)**
Reelin; mean (SD)	0.0014 (0.0009)	0.0016 (0.00089)
ApoER2; mean (SD)	29630.7 (14988.7)	37891.2 (17737.5)*
VLDLR; mean (SD)	103007.8 (43227.4)	112289.5 (39384.3)

ApoE: apolipoprotein E; GM: grey matter; ApoER2: apolipoprotein E receptor 2; VLDLR: very low-density lipoprotein receptor. *p = .09; **p < .05

Table 2.4 ApoE, cholesterol, reelin methylation, reelin, ApoER2, and VLDLR mRNA levels

There was no relationship between apoE, cholesterol, or mRNA levels and age at onset, duration of illness, or death by suicide in the psychiatric groups. Similarly, history of alcohol or drug use had no relationship with apoE, cholesterol in grey matter, methylation, or mRNA levels, the exception being cholesterol in white matter and alcohol use ($r = -.24$; $p = .04$). There were no differences in cholesterol or apoE levels when groups were stratified into violent and non-violent suicides.

Univariate ANOVA with dependent variable *RELN* promoter region methylation levels, and between-group factor diagnosis (two levels: SCZ, CTRL) revealed a statistically significant difference in *RELN* promoter region methylation, which was higher in SCZ (mean SCZ = .030 vs. mean CTRL = .012), $F (1, 69) = 5.96$, $p = .017$, $\eta = .082$).

Univariate ANCOVA (covariate age) with ApoER2 mRNA levels as the dependent variable and between-group factor diagnosis (two levels: SCZ, CTRL) revealed a non-significant trend for a main effect of diagnosis with lower levels of apoER2 mRNA in SCZ compared to CTRL (mean SCZ = 30,212 vs. mean CTRL = 37,412), $F (1,62) = 3.0$; $p = .09$; $\eta = .048$. There was a trend in the relationship of the covariate age and apoER2 level, $F (1, 62) = 3.5$, $p = .08$).

Univariate ANOVA with dependent variables cholesterol levels, or VLDLR mRNA, and between-group factor diagnosis (two levels: SCZ, CTRL) revealed no statistically significant main effect of diagnosis. Univariate ANCOVA with dependent variable apoE levels (covariate storage time), and between-group factor diagnosis (two levels: SCZ, CTRL) revealed no statistically significant main effect of diagnosis. Univariate ANCOVA (covariates brain pH and storage time) with dependent variable reelin mRNA levels as the between-group factor diagnosis (two levels: SCZ, CTRL) revealed no statistically significant main effect of diagnosis.

2.3.3 *APOE* allele effect on apoE, cholesterol, reelin methylation, reelin, ApoER2, VLDLR mRNA levels

Univariate ANCOVA (storage time as a covariate) with apoE levels as the dependent variable and between-group factor *APOE* genotype (three levels: *APOE*-ε2 carriers, *APOE*-ε3/ε3, and *APOE*-ε4 carriers; one *APOE*-ε2/ε4 excluded) revealed an effect of genotype after controlling for storage time $F(2,67) = 5.3$; $p < .001$; $\eta = .15$. *Post-hoc* contrasts (Sidak's corrected) revealed **lower**[l] apoE levels in grey matter in *APOE*-ε3/ε3 compared to *APOE*-ε2 (marginal mean *APOE*-ε2 = 20.2 vs. marginal mean *APOE*-ε3/ε3 = 80.9; $p = .01$) and *APOE*-ε4 compared to *APOE*-ε2 (marginal mean *APOE*-ε4 = 88.4 vs. marginal mean *APOE*-ε2 = 20.2; $p = .02$). ApoE levels in *APOE*-ε4 compared to *APOE*-ε3 were similar. The covariate, storage time, was significantly related to the apoE levels, $F(1, 67) = 15.5$, $p < .001$). ApoE levels were as follows: *APOE*-ε2 > (*APOE*-ε3/ε3 ~ *APOE*-ε4).

Univariate ANCOVA (brain pH as a covariate) with reelin methylation levels as the dependent variable and between-group factor *APOE* genotype (three levels: *APOE*-ε2 carriers, *APOE*-ε3/ε3, and *APOE*-ε4 carriers; one *APOE*-ε2/ε4 excluded) revealed an effect of *APOE* genotype after controlling for brain pH time $F(2,68) = 6.3$; $p < .01$. *Post-hoc* contrasts (Sidak's corrected) revealed higher reelin methylation levels in *APOE*-ε3/ε3 compared to *APOE*-ε2 (marginal mean *APOE*-ε2 = - .004 vs. marginal mean *APOE*-ε3/ε3 = .03; $p = .002$). Reelin methylation showed a trend to higher levels in *APOE*-ε4 compared to *APOE*-ε2 (marginal mean

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[l] Please note enzyme-linked immunosorbent assay (ELISA) data values (protein amount required to produce a fixed optical density of 0.4) are inversely related to the amount of antigen present; therefore, lower values represent higher apoE concentrations.

APOE-ε4 = .019 vs. marginal mean *APOE*-ε2 = -.004; p = .1). Reelin methylation in *APOE*-ε4 compared to *APOE*-ε3 was similar. The covariate, brain pH, was significantly related to the reelin methylation, F (1, 68) = 5.1, p < .03). Methylation levels of reelin promoter region were as follows: (*APOE*-ε3/ε3 ~ *APOE*-ε4) > *APOE*-ε2.

Univariate ANCOVA (brain pH as a covariate) with reelin mRNA levels as the dependent variable and between-group factor *APOE* genotype (three levels: *APOE*-ε2 carriers, *APOE*-ε3/ε3, and *APOE*-ε4 carriers; one *APOE*-ε2/ε4 excluded) revealed a trend for an effect of genotype after controlling for brain pH and storage time F (2,67) = 2.3; p = .01; η = .07. *Post-hoc* contrasts (Sidak's corrected) revealed a trend toward higher reelin mRNA levels in *APOE*-ε2 compared to *APOE*-ε3/ε3 to (marginal mean *APOE*-ε2 = .002 vs. marginal mean *APOE*-ε3/ε3 = .001; p = .1). Reelin mRNA levels were similar in the rest of contrasts. The covariates brain pH and storage time were both significantly related to the reelin mRNA levels, brain pH F (1, 67) = 8.1, p = .006, η = .11 and storage time F (1, 67) =10.4, p = .002, η = .14. Reelin mRNA levels trended to *APOE*-ε2 > *APOE*-ε3/ε3.

2.3.4 ApoE and cholesterol in grey and white matter

An independent-samples t-test was conducted to compare grey and white matter apoE levels. There was a significant difference in the levels of apoE in grey matter (M = 70.7, SD = 72.1) compared to white matter (M = 194.4, SD = 105.2); t (136) = 8.06, p < .001. ApoE levels were higher is grey matter compared to white matter.

An independent-samples t-test was conducted to compare grey and white matter cholesterol levels. There was a significant difference in the levels of cholesterol for grey matter (M = 154.7, SD = 70.2) and white matter (M = 335.5, SD = 56.2); t (120.2) = 16.7, p < .001. Cholesterol level was higher in white matter compared to grey matter.

Linear correlational analyses were conducted to explore the relationship of apoE and cholesterol in grey and white matter. In both grey and white matter, apoE and cholesterol were negatively correlated (grey matter, $r = -.44$; $p < .001$ and white matter, $r = -.3$; $p = .01$), with higher levels of apoE being associated with lower amount of cholesterol. In addition, when groups were stratified into SCZ and CTRL the association between levels of apoE and cholesterol was statistically significant, and stronger, only in CTRL in both grey and white matter (grey $r = -.56$; $p < .0001$; white matter, $r = -.37$; $p = .03$), where higher levels of apoE were associated with lower levels of cholesterol.

2.4 Discussion

2.4.1 ApoE, cholesterol, *RELN* promoter region methylation level, reelin mRNA, apoER2 mRNA, and VLDLR mRNA levels in SCZ

The main findings observed were the increased levels of methylation of the *RELN* promoter region and a non-significant trend for lower levels of apoER2 mRNA in SCZ patients. There were no statistically significant differences in apoE, cholesterol, reelin mRNA, and VLDLR mRNA.

A potential role of reelin in the etiopathogenesis of SCZ was rapidly postulated upon the discovery of reelin's critical role on neuronal migration during development as well as its relevance in modulating NMDA and AMPA receptor activity in the adult brain[254,256,290]. Thereafter, postmortem studies in human brain showed reduced *RELN* mRNA levels in several brain areas including BA9, 10, 22, hippocampus, caudate, and cerebellum[268,269,291,292], although others only found decrease in the left BA9 but not on the right BA9 or bilaterally in BA1-3, 22, 28, 46, or hippocampus (CA4)[293]. Initially, the report of increased methylation, a putative epigenetic regulation mechanism, of the promoter region of *RELN* in patients with SCZ provided

a potential mechanistic explanation for decreased levels of expression of reelin[271,272,294]. The current experimental results are in partial agreement with prior literature, as an increase in the level of methylation of the promoter region of *RELN* in SCZ was observed, even though this was not associated with a statistically significant decrease of reelin expression in the same brain region. A number of reasons may explain these partially divergent findings. First, research using precise techniques to investigate the expression of reelin in specific neuronal populations and in concrete areas within the cerebral cortex have shown that decreased reelin expression and increased methylation of the promoter region in SCZ in the cerebral cortex may preferentially occur in GABAergic interneurons in layer I whereas layer V would not show such differences[295]. Minor heterogeneity of cortex sampling when measuring the entire cortex may have resulted in increased variability of reelin expression. Second, we found that both storage time and brain pH were strongly correlated to reelin expression and remained highly significant in the ANCOVA models. This might have acted as a confounder and increased the dispersion of data and decreased statistical power. Third, a pivotal argument posed in Chapter 1 is that our current diagnostic label of "Schizophrenia" is a syndromic label, and therefore our sample of 35 SCZ patients may have been comprised of a subgroup of patients who indeed have abnormal reelin expression as their underlying etiopathogenesis while other patients' syndromic presentation may be due to other etiopathogenic mechanisms.

Our finding of a non-significant trend of increased expression of VLDLR in SCZ would be convergent and further those of Suzuki and colleagues. They found decreased expression of VLDLR in peripheral lymphocytes in antipsychotic naïve SCZ patients, and VLDLR expression subsequently increased after initiation of neuroleptic treatment in peripheral lymphocytes[287]. The

increased expression of VLDLR in the brain in our sample may be explained by the chronic exposure of antipsychotics in our sample of chronically treated SCZ patients.

In contrast to our findings, apoE levels were higher in the prefrontal cortex (BA 9 and 46) in SCZ in two other studies[126,127]. Several possible reasons might account for the discrepancy between our data and those of the two previous studies. First, sample size, clinical, and demographic characteristics were different. The proportion of men was higher in our sample (n = 35 per group, 74% men in both) compared to Dean et al. (SCZ n = 23, 53% men; CTRL n = 45, 57% men) and Digney et al. (SCZ n = 18, 53% men; CTRL n = 17, 50% men). Also our sample was younger (mean age = 43) compared to that of Dean et al. (mean age = 51) or Digney et al. (mean age = 47; computed from table 1 in[126]). Second, the amount of antipsychotic medication might have been different as different methods to report antipsychotic dose were used. Whereas Digney et al. and Dean et al. both reported the mean final recorded antipsychotic CPZ equivalent (461mg/d and 559mg/d respectively) we reported lifetime CPZ equivalent dose. Third, in the previous studies apoE was only measured in the left hemisphere, whereas our sample randomly selected from either hemisphere. Last, the method to quantify apoE was also different. Current data were measured by means of an ELISA assay, which targets the protein of interest in its native conformational state, whereas the previous studies used western blotting, which targets the protein's epitope in an unfolded state. In agreement with our previous study of visual association cortex[236], we found that cholesterol levels were not significantly different in SCZ.

2.4.2 *APOE* allele effect on apoE, cholesterol, reelin methylation, reelin, ApoER2, VLDLR mRNA levels

In grey matter, apoE levels were significantly higher in *APOE*-ε2 carriers compared to either *APOE*-ε3 or *APOE*-ε4 carriers. This result suggests a genotypic regulation of apoE. Our

results are consistent with prior studies in animal models with humanized apoE expression demonstrating that apoE levels in the brain followed a gradation *APOE*-ε2 > *APOE*-ε3 > *APOE*-ε4[265]. It is not entirely clear what mechanisms may underlie this differential level, but it has been shown that apoE4 is more prone to remain in a partially unfolded tertiary conformation, making it less efficient, and potentially more vulnerable to degradation[296]. Similar investigations in AD have yielded inconsistent data due to the confounding effect of apoE binding to amyloid deposits.

The association of *APOE*-ε3 genotype to higher levels of reelin methylation is a novel finding and contribution to the literature. Although the data cannot confirm a causality link, they do indicate cross talk between different molecular players in the LDLR signal transducing system. In addition, the non-significant trend for higher reelin levels in *APOE*-ε2 genotype is internally consistent with lower methylation of the promoter regulatory region of *RELN* in *APOE*-ε2 and provides converging evidence to support regulatory cross talk between *APOE*/apoE and RELN/reelin. Prior research showed that the signalling pathway involving apoE and reelin as ligands to the LDLR receptors apoER2 and VLDLR is subject to regulation as a system[250]. As such, *APOE* genotype association to the methylation of the regulatory region of reelin and reelin levels further supports the hypothesis of the apoE/reelin-apoER2/VLDLR system being under complex and concerted regulation. It can be concluded that studies in humans addressing questions at a systems level to explore complex interactions are not only worthwhile, but a rather necessary pursuit to illuminating the etiology of schizophrenias.

2.4.3 Insights about the physiology of *APOE*/apoE and cholesterol in the human brain

The current results on the levels of cholesterol and apoE in grey vs. white matter represent a novel finding and are relevant contributions to our understanding of cholesterol

metabolism in the human brain. While cholesterol levels were higher in white matter than in grey matter, apoE amounts were greater in grey matter than in white matter. Since a large proportion of brain cholesterol (approximately 70%) is present in myelin, our data showing higher levels of cholesterol in white matter is consistent with prior literature reporting a cholesterol ratio of approximately 0.45:1 between grey and white matter[297,298], similar to that seen in the present study. In contrast, the significantly lower levels of apoE in white matter may be interpreted as an indication of different metabolic *dynamics* of cholesterol in either tissue pool. The brain depends on a highly efficient apolipoprotein-dependent recycling mechanism to maintain cholesterol homeostasis. Prior research shows that cholesterol turnover in the myelin pool is extremely low in the non-pathologic adult brain[279], indicating that cholesterol in myelin could be conceptualized as a relatively *static* pool of cholesterol. Our results showing higher levels of apoE in grey matter may reflect the role of apoE as the main cholesterol transport mechanism in the brain. This would be likely as grey matter is characterized by a wide array of dynamic cellular activities that require cholesterol trafficking such as neurotransmitter release, synaptic plasticity, and cellular membrane homeostasis. Cholesterol in grey matter may be conceptualized as a *dynamic* pool of cholesterol *in itinere*[m]. Alternatively, other apolipoproteins may be involved in cholesterol transport in white matter[298].

In grey, and to a lesser extent, white matter we observed an inverse relationship between apoE and cholesterol levels. The inverse association may seem counterintuitive. However, we measured free cholesterol (i.e. that bound to membranes) whereas cholesterol is transformed into the esterified form once loaded into the core of apolipoproteins[299]. It seems plausible that when

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[m] *In itinere*, latin expression meaning “in transit” or “on a journey”.

apoE levels are high more cholesterol is in the esterified form rather than the free form. In addition, the CTRL group showed a strong association between cholesterol and apoE, which was not present in the SCZ group. This finding may reflect a pathological process occurring in SCZ, which interferes with the lipidation of apoE.

2.4.4 Limitations

There are some intrinsic limitations in our study that need to be addressed. The main intrinsic limitation of the study is that human postmortem data capture the end stage and cumulative effect of a wide array of variables. This provides us with the last snapshot of a sequential chain of events; an analogy would be that of taking a picture of a room where a party has taken place. We may see objects in a particular configuration and we may be able to infer whether there were more or less people, but we cannot be certain of the dynamic events leading to that final snapshot. Similarly, we are not able to experimentally control for any variables or have detailed information about potential confounding variables. One such important confounding factor is exposure to antipsychotic medication. Although the information we have is circumscribed, we observed no correlation between apoE or cholesterol levels and lifetime antipsychotic dose, and there were no differences in apoE or cholesterol values between groups treated with atypical antipsychotics, typical antipsychotics, or no antipsychotic treatment previous to death. Our data do not agree with those of a previous study that reported a significant reduction in apoE levels in grey matter in rats with rodent APOE gene and rodent apoE isoform treated with haloperidol[127]. Although animal studies are informative, it is important to bear in mind differences compared to human neurophysiology when translating research results. Rodents do not have the three common human allele variants for *APOE* and this difference has

an impact on apoE and cholesterol metabolism in rats. The effect of antipsychotic treatment on apoE and cholesterol in human brain remains open to further research.

A second potentially important factor is the presence of lipid-lowering medications in these patients. Risk for cardiovascular disease and dyslipidemia is elevated in patients with SCZ relative to the general population[300,301]. Unfortunately, information on whether patients were prescribed statins or other lipid-lowering drugs before death was unavailable to us. Although animal studies indicate that administration of high doses of simvastatin or pravastatin may change total brain cholesterol levels[302,303], a study of human volunteers showed that administration of a high dose of either simvastatin or pravastatin did not change the plasma 24(S)-hydroxycholesterol to cholesterol ratio, a surrogate marker of brain cholesterol homeostasis[304]. In addition, moderate or heavy past or present alcohol use was reported in a significant proportion of the psychiatric patients. In this group of patients, we found a significant deficit in cholesterol levels in white matter but not in grey matter relative to patients reported to have no or only social alcohol use. This finding is contrary to that of Olsson and colleagues who reported no change in cholesterol levels in either grey or white matter in a small series of individuals with alcoholism[305]. Although the sample size for a postmortem study in human brain is very high the number of different *APOE* genotypes per group is relatively small and precluded examination of the relation between apoE and cholesterol levels between groups stratified by genotype. Lastly, the pH of brain samples was lower in SCZ compared to CTRL. Although this variable was added as control variable in statistical analyses where appropriate, this difference may have masked potential findings. Also, the different brain pH values between groups may have been related to differences in agonal stages, although there were no differences in pH or

other measures between those SCZ patients who died of completed suicide or other causes of death.

Chapter 3: Apolipoprotein varepsilon 4 and structural neuroimaging in schizophrenia[n]

3.1 Introduction to chapter 3

3.1.1 MRI hippocampus abnormalities in schizophrenia

Schizophrenia (SCZ) is a disorder characterized by the presence of pervasive, profound, and ongoing cognitive impairment as well as episodic psychotic symptoms, such as delusions and hallucinations[21,306]. The disorder is associated with severe functional impairment and suffering in both patients and their families[307]. Although its precise molecular and cellular neurobiological underpinnings remain elusive, neuroimaging tools have provided a wealth of evidence with regards to macroscopic neuroanatomical[217] and functional abnormalities[308].

Reduced hippocampal volume is one of the most robust brain abnormalities demonstrated in SCZ. Reduced hippocampal volume has been shown in both retrospective meta-analysis[217] as well as in large consortium initiatives using prospective meta-analytic methodology[216]. The effect sizes reported are large and consistent in both reports d = − 0.52 (− 0.60 to − 0.44; 95% CI; [217]) and d = − 0.46 (− 0.58 to − 0.34; 95% CI; [216]) respectively. Volume deficits are seen even in the early stages of illness and in antipsychotic naïve patients, with similar reported effect sizes (d = − 0.43; − 0.63 to − 0.24; 95% CI; [217]), indicating that smaller hippocampal volume compared to CTRL is present in early stages of the illness and is not a mere by-product of illness

[n] A version of this chapter is in preparation for submission

progression or antipsychotic treatment. A meta-analysis of 27 neuroimaging studies in SCZ looking at the progression of brain structural abnormalities over a span of 1 to 10 years found no differences between patients and CTRL in the change of volume of the hippocampus over time[309].

3.1.2 *APOE* and hippocampus abnormalities

The Apolipoprotein E gene (*APOE*) plays a critical role in synaptogenesis, neurite outgrowth, and membrane repair and maintenance[57,60,246,249]. The *APOE* gene has three main polymorphisms (*APOE*-ε3, *APOE*-ε4, and *APOE*-ε2), and the *APOE*-ε4 allele is the main genetic risk factor for late onset Alzheimer's disease[310]. *APOE*-ε4 is associated with bilateral smaller hippocampi both in preclinical stages of AD as well as after onset of AD[311]. Furthermore, there is a dose dependent relationship where *APOE*-ε4 homozygotes have smaller hippocampal volumes than heterozygous[312] and decrease in hippocampal volume has been associated with verbal memory impairment, linking brain structure abnormalities to clinical phenotype[312]. Further to the potential role of *APOE*-ε4 in hippocampal volume abnormalities, in AD there is evidence of synergistic effects of β-amyloid load and *APOE*-ε4 whereby those with higher β-amyloid load and *APOE*-ε4 present with more pronounced hippocampal volume reductions[312]. Hata and colleagues investigated the effect of *APOE* gene on hippocampal volumes on 21 patients with chronic SCZ (mean age 34 yrs, APOE-ε4 carriers = 7) and found a nonsignificant trend for *APOE*-ε4 carriers having smaller right hippocampus[101].

3.1.3 Aims and hypotheses

Aim 1. To investigate the effect of the *APOE*-ε4 allele on hippocampal volume in first-episode psychosis.

Aim 2. To investigate the relationship between hippocampal volume and verbal memory in first-episode psychosis.

Hypothesis 1. *APOE*-ε4 will be associated with lower hippocampal volume in first-episode psychosis.

Hypothesis 2. Hippocampal volume will be associated with verbal memory impairment.

3.2 Material and methods

3.2.1 Methods

The research protocol was approved by the University of British Columbia and Vancouver Coastal Health Authority Clinical Research Ethics Boards, and was in accordance with Tri-Council policy. Written informed consent was obtained from all participants after the procedures had been fully explained. Participants were recruited from a longitudinal study from an Early Psychosis Intervention program serving a catchment area population of 640,000 in the Vancouver area. All patients referred to the program were approached to enter the study. Participants had less than 8 weeks of total lifetime exposure to antipsychotic treatment at the time of cognitive assessment and brain imaging.

Inclusion criteria for patients for this study were presentation of DSM-IV defined psychotic disorders, and fluency in English. Exclusion criteria were i) history of significant head injury defined by loss of consciousness greater than 5 minutes, ii) history of neurodevelopmental disorder or central nervous system infection, iii) history of inhalant intoxication, iv) inability to

provide full written consent, and v) IQ less than 75. Inclusion and exclusion criteria were similar for healthy volunteers, with additional exclusion for history of major psychiatric disorder.

Diagnoses were based on the Structured Clinical Interview for DSM-IV, a review of clinical case notes, and interviews with one or more family members. This information was presented at a case conference, and two psychiatrists and a clinical psychologist made a consensus diagnosis. A detailed review of past and current substance use was obtained at baseline and follow-up. The Positive and Negative Syndrome Scale (PANSS;[313]) estimated psychiatric symptom severity at baseline and follow-up. A five-factor solution was computed at baseline and follow up based on a meta-analysis of FES[314]. CPZ equivalents were computed according to Woods and colleagues[315]. Level of functioning was indexed at baseline using the Social and Occupational Functioning Assessment Scale (SOFAS;[273], clinical global impression (CGI;[316]) and the Role Functioning Scale (RFS;[317]).

3.2.1.1 Participants

The participants were 68 FEP patients and 47 control subjects (CTRL). Within the FEP group diagnoses were SCZ (37), schizoaffective disorder (12), affective psychosis (13) and psychosis, not otherwise specified (7). FEP and CTRL groups were similar in mean age, sex, ethnicity distribution, and prevalence of *APOE*-ε4 carriers (Table 3.1).

A summary of demographic and clinical characteristics comparing the FEP APOE-ε4 carriers group (FEP- APOE-ε4+) versus the FEP non- APOE-ε4 carrier group (FEP- APOE-ε4-) at baseline is shown in Table 3.2. In summary, both groups were similar in mean age, mean years of education and sex distribution. In addition, both groups had similar premorbid and current estimated IQ. The two groups were similar with respect to symptom severity, level of functioning, and medication status.

	FEP (n = 68)	CTRL (n = 47)
Male:Female (%Male)	41:27 (60.3%)	22:25 (46.8%)
Age, yrs; mean (SD)	20.7 (5.5)	23.9 (8.9)**
Ethnicity; Cau:Asi:Other (% Cau)	47:12:9 (69.1%)	33:10:4 (70.2%)
APOE-ε4 carriers+; n (%)	25 (36.8%)	11 (23.4%)
Education; yrs; mean (SD)	11.5 (1.8)	13.9 (2.4)*
NAART FSIQ; mean (SD)	100.3 (8.3)	108.3 (7.7)*
K-BIT IQ; mean (SD)	95.3 (10.6)	109.2 (8.7)*

+One APOE2/4 was excluded; *p < 0.01; **p < 0.001
Abbreviations: FEP: First Episode Psychosis; CTRL: Control; yrs: years; S: single; P: Partner; Cau: Caucasian; Asi: Asian; NAART: FSIQ North American Reading Test Full Scale IQ; K-BIT: IQ Kauffman brief Intelligence Test IQ.

Table 3.1 Demographic characteristics and premorbid and current intelligence quotient (IQ) of the sample

FES sample only	**Non-*APOE*-ε4 (n = 43)**	***APOE*-ε4 (n = 25)**
Male: Female (% Male)	26:17 (60.5%)	15:10 (60%)
Age in years; mean (SD)	21.0 (6.3)	20.3 (4.1)
Ethnicity; Cau:Asi:Other(%Cau)	29:9:5 (67.4%)	18:3:4 (72%)
Education in years; mean (SD)	11.3 (1.7)	11.8 (1.9)
NAART; mean (SD)	100.6 (8.0)	99.8 (9.0)
K-BIT IQ ; mean (SD)	96.4 (8.0)	93.5 (14.0)
Diagnoses, SCZ:SCZ_A:AfP:other (% SCZ)	25:8:6:4 (58.1%)	12:4:6:3 (48%)
PANSS Scores; mean (SD) Positive Negative General Total	 17.0 (6.0) 16.9 (5.0) 38.3 (7.3) 72.2 (14.8)	 17.1 (6.2) 18.2 (7.0) 39.2 (14.8) 74.5 (19.0)
CGI; mean (SD)	4.0 (0.7)	4.0 (1.1)
Onset of symptoms: Age First Psychosis, mean (SD) DUP (mo); mean (SD)	 18.9 (5.8) 29.7 (43.8)	 19.1 (5.0) 16.6 (23.2)
Medications: Ris/Ola/other/missing CPZ; mean(SD) AP naïve; n (%)	 14/11/2/4 190.7 (182.9) 12 (27.9%)	 12/6/0/1 125.0 (72.7) 6 (24%)

Abbreviations: NAART: North American Reading Test; K-Bit IQ: Kauffman Brief Intelligence Test IQ; SCZ: Schizophrenia; SCZ-A: Schizoaffective; AfP: Affective psychosis; DUP: Duration of untreated psychosis; Ris: Risperidone; Ola: Olanzapine; AP: Antipsychotic; PANSS: Positive and Negative Syndrome Scale; CGI Clinical Global Impression;

Table 3.2 Clinical characteristics of first episode psychosis patients, non-*APOE*-ε4 carriers vs. *APOE*-ε4 carrier

3.2.1.2 *APOE* genotyping

Genotyping of the *APOE* rs7412 and rs429358 polymorphisms was performed using standard ABI (Applied Biosystems Inc., Foster City, CA, USA) SNP Genotyping Assays. Genotype calls were performed manually. Laboratory personnel blind to demographic and phenotypic information independently verified the results.

To ensure quality control, 10% of the samples were randomly re-genotyped with a 100% concordance rate.

3.2.1.3 Image acquisition and processing

All patients and CTRL received an MRI brain scan at presentation and 48 patients (*APOE*-ε4 carriers = 18, non *APOE*-ε4 = 30) and 29 CTRL (*APOE*-ε4 carriers = 7, non-*APOE*-ε4 carriers = 22) were available for rescanning after a mean interval time of 44 weeks. Scans at presentation and follow-up were acquired using a GE Sigma EXCITE 1.5T scanner with 1.5 mm contiguous slices and acquisition matrix size set at 256 × 256. All were acquired using T1-weighted three-dimensional FSPGR IR prepped series using an 8-channel receive headcoil (TR = 11.2 ms, TE = 2.1 ms, FOV = 26 cm2, flip angle = 20°, voxel size = 1.01 mm3). DICOM images were converted to standard NIfTI format with MICRON dcm2nii freeware. FreeSurfer shareware (v.4.3; http://surfer.nmr.mgh.harvard.edu/) was used to automatically parcellate the hippocampi after alignment with FreeSurfer's standard brain mask[318]. Our group has previously described manual vs. automated segmentation agreement in this population (ICC = 0.91;[319]).

3.2.1.4 Neuropsychological assessment

Trained research assistants under the supervision of a clinical psychologist conducted neuropsychological assessments. Participants were administered a comprehensive battery of standardized tests including measures of memory, motor functioning and intellectual abilities.

Premorbid intellectual functioning was estimated at baseline with the North American Adult Reading Test (NAART;[320]). Current IQ was estimated at baseline with the Kaufman Brief Intelligence Test (K-BIT;[321]). Verbal memory was assessed using the California Verbal Learning Test 2nd Edition (CVLT-II) immediate recall trials 1-5[322].

3.2.1.5 Statistical Analyses

Statistical analyses were performed with SPSS (version 24.0; IBM corp). Data Exploratory Analysis was used to ascertain the characteristics of the distribution of the variables, including testing for normality. Chi-square tests were used to test the null hypothesis of equality of proportions in different groups (two levels of sex, men and women; three levels of ethnicity, Caucasian, Asian, and other; four levels of diagnoses, SCZ, schizoaffective, affective psychosis, and other; two levels of *APOE*-ε4 status, *APOE*-ε4 carriers and non *APOE*-ε4 carriers; four levels of type of medication (naïve, olanzapine, risperidone, other). Hardy–Weinberg equilibrium of *APOE* alleles was assessed using an exact Fisher's test. Independent t-tests were used to test the null hypothesis of equal means in different independent groups for normally distributed variables age, years of education, NAART, K-BIT, and PANSS total score. The Mann-Whitney U statistic was used to test the null hypothesis of equal means in different independent groups for non-normally distributed variables CGI, age at first decline, age at first psychotic symptoms, duration of untreated psychosis, and CPZ equivalent dose at time of scan.

Correlational analyses were used to explore the relationship between hippocampal volume and brain volume, sex, age, duration of untreated psychosis, age of first decline, CPZ equivalent dose, CGI, PANSS, RFS, NAART, K-Bit, and CVLT 1-5. Partial correlations with brain volume as a covariate were also run on the same variables to ascertain the impact of adjusting for brain volume. Correlation analyses were not corrected for multiple comparison [323]

An omnibus mixed-model ANCOVA was used with hippocampal volume as a within factor (left; right), and diagnosis (FEP, CTRL) and *APOE*-ε4 status (*APOE*-ε4 carrier; *APOE*-ε4 non-carrier) as independent variables and brain volume as a covariate. Sex and age were not correlated to hippocampal volume and subsequently not entered in the models. Follow up two-way ANCOVA was used with total hippocampal volume as dependent variable, diagnosis (FEP, CTRL) and APOE-ε4 status (*APOE*-ε4 carrier; *APOE*-ε4 non-carrier) as independent variables and brain volume as a covariate was performed.

SPSS MIXED procedure was used to perform a mixed-model repeated measures (MMRM) analysis of hippocampal volume, diagnosis and *APOE*-ε4 status over time. For fixed effects, diagnosis (FEP vs. CTRL) and *APOE*-ε4 status (*APOE*-ε4 carriers vs. non-*APOE*-ε4 carriers) were entered as between-subjects, and time (baseline vs. follow up) as within-subjects factor. None of the potential double interactions or the triple interaction Diagnosis by *APOE*-ε4 by Time were significant. The most parsimonious model did not include either of those terms. Brain volume was entered as a covariate. An unstructured covariance matrix was assumed. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. A multiple linear regression was computed with CVLT trials 1-5 score as the predicted outcome and diagnosis (0 = CTRL, 1 = FEP), *APOE*-ε4 status (*APOE*-ε4 carriers = 0, *APOE*-ε4 non-carriers = 1), and hippocampal volume as predictors. Force enter procedure was used. All reported values are marginal means and standard deviations. These values were obtained after adjusting for covariates.

3.3 Results

3.3.1 Hippocampal volume and *APOE*-ε4 status

The omnibus mixed-model ANCOVA did not reveal statistically significant differences between right and left hippocampal volume, therefore subsequent model used total hippocampal volume as the dependent variable and brain volume as a covariate. Two-way ANCOVA (Figure 3.1) revealed significantly smaller hippocampal volume in patients than CTRL (FEP = 8444.1 vs. CTRL = 8603.7; F (1,111) = 4.3; p = .04, d = 0.23) with a main effect for *APOE*-ε4 status (*APOE*-ε4 carriers = 8312.6 vs. non *APOE*-ε4 carriers = 8734.7; F (1,111) = 6.8, p = .01, d = -0.52) and a trend for a diagnosis-by-allele interaction (F (1,111) = 2.9, p = .09).

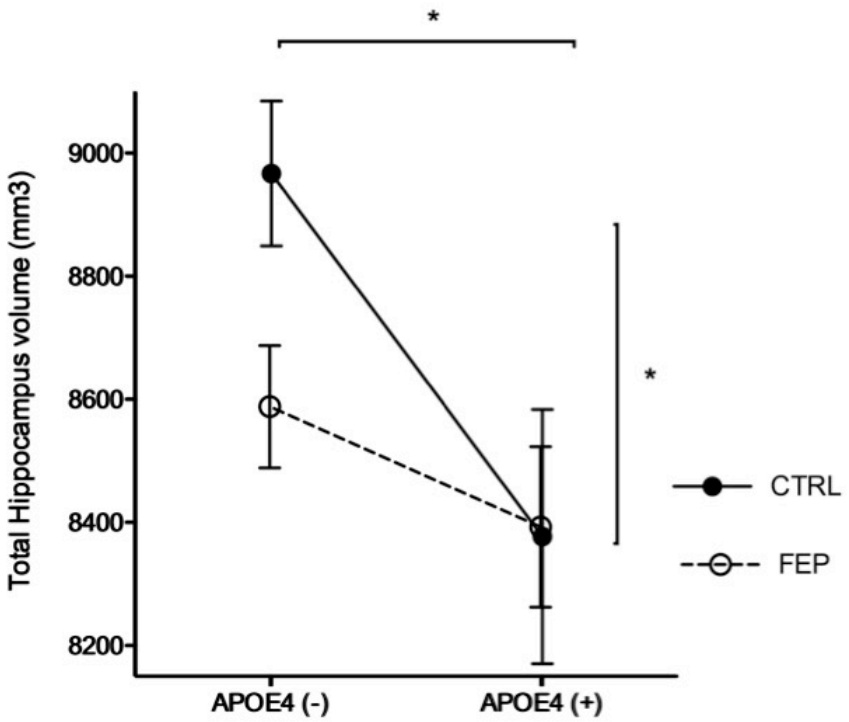

Figure 3.1 Smaller hippocampal volume in FEP

3.3.2 Hippocampal volume change over time and *APOE*-ε4 status

Mixed-model repeated measures analyses revealed a main effect of diagnosis on hippocampal volume (FEP = 8441.8 vs. CTRL = 8693.8; F (1, 112.0) = 3.96; p = .049) as well as a main effect of APOE-ε4 on hippocampal volume (*APOE*-ε4 carriers = 8397.1 vs. *APOE*-ε4 carriers non-carriers = 8738.5; F (1, 111.8) = 6.5; p = .012) with no effect of Time (Baseline = 8566.5 vs. Follow up = 8569.1; F (1, 75.9) = 0.12; p = .9). The effect of brain volume was statistically significant (F (1, 132.6) = 71.4, p < .001). See Figure 3.2.

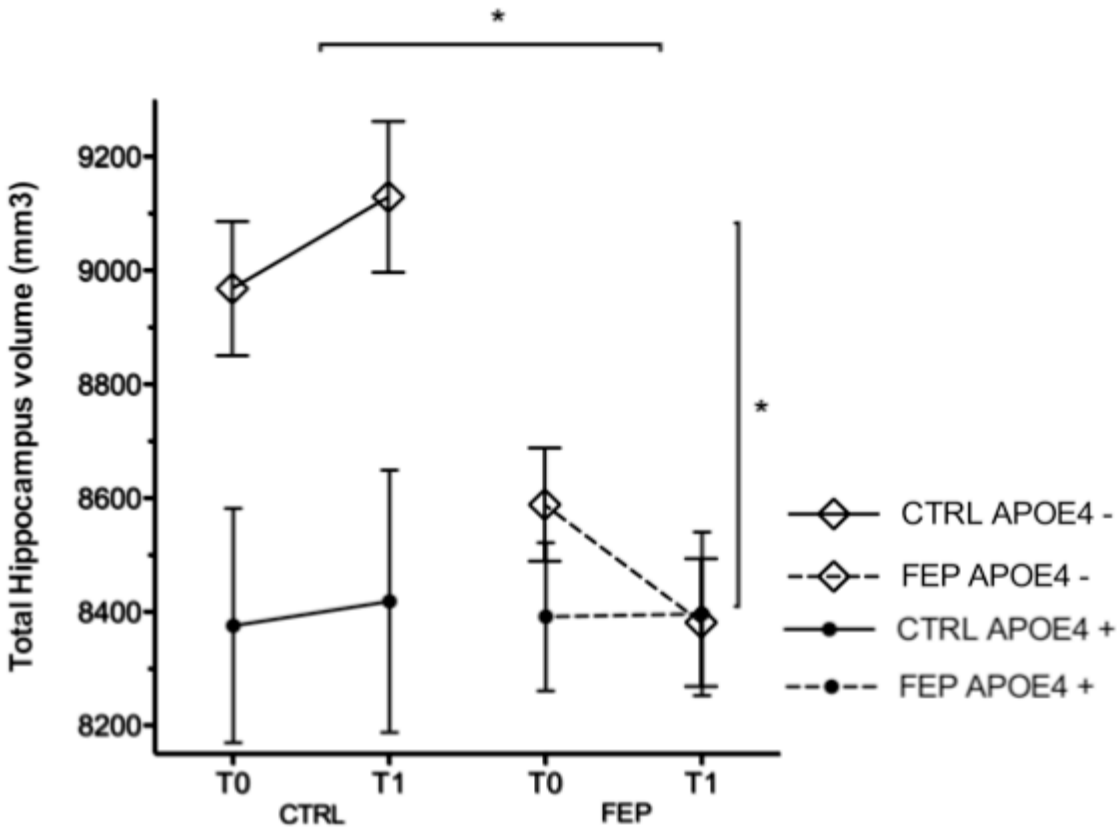

Figure 3.2 Hippocampal volume change over time in FEP and CTRL

3.3.3 Verbal Memory and clinical outcomes

Mean premorbid IQ as well as estimated current IQ was similar in both FEP *APOE*-ε4 carriers and FEP *APOE*-ε4 non-carriers (Table 3.1). In order to test the contribution of hippocampal volume on verbal memory, a multiple linear regression was calculated to predict verbal memory (i.e. CVLT 1-5) based on dependent variables diagnosis, *APOE*-ε4 status, and hippocampal volume. A significant regression was found ($F(3,112) = 13.4$, $p < .001$), with an R^2 of .264. Participant's predicted verbal memory was equal to 34.4 – 13.4 (Diagnosis) - .41 (*APOE*-ε4 status) + .001 (Hippocampal volume), where Diagnosis is coded as 0 = CTRL, 1 = FEP, *APOE*-ε4 status is coded as *APOE*-ε4 carriers = 0, *APOE*-ε4 non-carriers = 1, and hippocampal volume measured in mm^3. Verbal memory scores increased .001 for each mm^3 of hippocampal volume, CTRL scored 13.4 points more than FEP, and *APOE*-ε4 non-carriers scored .041 points more than *APOE*-ε4 carriers. Only diagnosis was a significant predictor of verbal memory performance. In addition, partial correlation was used between verbal memory and hippocampal volume after controlling for brain volume and sex, but were not statistically significant ($p > 0.2$). Partial correlation was also used to explore the relationship between hippocampal volume and clinical outcomes with brain volume as a covariate. Partial correlations between hippocampal volumes, PANSS total score, RFS, and CGI did not reveal any statistically significant relationships.

3.3.4 Assessing potential confounding variables: age, diagnosis, duration of untreated psychosis diagnosis and medication

Partial correlations between age and hippocampal volumes were not statistically significant for the total patient or control samples or within either of the *APOE*-ε4 status.
To assess the effects of diagnosis, the primary analysis was repeated after limiting the patient sample to those with SCZ or schizoaffective disorder. This ANCOVA produced similar findings to the overall results with significantly smaller hippocampi in patients than CTRL (FEP = 8431.8 vs. CTRL = 8606.7; F (1,91) = 4.5 p = .04, d = 0.22) with a main effect for *APOE*-ε4 status (*APOE*-ε4 carriers = 8329.4 vs. non *APOE*-ε4 carriers = 8709.1; F (1,91) = 4.8, p = .03, d = 0.48) and a trend for diagnosis-by-allele interaction (F (1,91) = 3.3, p = .07). Duration of untreated psychosis (DUP) did not significantly differ as a function of *APOE*-ε4 status. In addition, non-parametric correlation between DUP and hippocampal volume did not reveal a significant effect (non-parametric).

The CPZ equivalent dose as a function of *APOE*-ε4 status was similar in both groups. In addition, the 18 patients who were antipsychotic naïve were compared to the 45 treated patients in order to assess the effects of brief antipsychotic exposure on hippocampal size. An ANCOVA between groups (antipsychotic naïve, treated) and *APOE*-ε4 allele (carrier, non-carrier) with brain volume as a covariate failed to detect significant differences in total hippocampal volume between antipsychotic naïve and treated patients and revealed no significant treatment-by-allele interaction (all p values > 0.30).

3.4 Discussion

3.4.1 *APOE*-ε4 is associated with smaller hippocampus

Results are convergent with existing literature on volume reductions of the hippocampus in SCZ and extend them to the spectrum of FEP. In addition, data also indicated a detrimental effect of the *APOE*-ε4 allele on hippocampal volume, and it would appear the effect of the disorder and *APOE*-ε4 are additive rather than synergistic as there is no disorder-by-*APOE*-ε4 interaction associated with reduction of hippocampal volume. The data did not support a lateralization effect of either the disorder or the *APOE*-ε4 allele, as the volume reduction was bilateral.

Of particular interest, *APOE*-ε4 was no longer beneficial in terms of hippocampal volume, in contrast to the findings reported in chapter 3 for verbal memory. In the context of hippocampal volume there appears to be no advantage of carrying the *APOE*-ε4 even in youth, and this would apply to both healthy volunteers and FEP subjects alike. A potential hypothesis to explain this observation is that it would be related to the pleiotropic effects of *APOE*, and particularly with it role during brain development. A study testing whether the *APOE*-ε4 allele is associated with decrease cell number or decreased neuropil density would explain the decrease in volume. Although the mechanism linking *APOE*-ε4 to hippocampal volumetric changes is beyond the scope of this work, these data do contribute to narrowing the timeline when hippocampal volumetric alterations occur. While our data revealed differences in a young adult sample, prior work in a large sample of adolescents did not report any hippocampal volume differences as a function of *APOE*-ε4 status[324]. This suggests the beginning of volumetric changes associated with *APOE*-ε4 status might be around late adolescence. Around this age critical events occur as part of brain development during adolescence such the fine-tuning of

excitatory/inhibitory synapses[325,326] and myelination[327]. Deviations from a normal developmental trajectory have been postulated as a potential mechanism to explain the onset of SCZ at this particular age. The preponderance of Apoliprotein E (ApoE, protein) levels in cortex as opposed to adjacent white matter as reported above in chapter 2 as well as its critical role in cholesterol homeostasis, synaptogenesis, and neurite outgrow, make apoE a prime candidate to play a role in morphometric differences. Furthermore, ApoE-ε4 has been shown to be less efficient in its function than other isoforms and this might have an impact in times of increased metabolic and remodeling demands, such as when developmental changes occur in the hippocampus, and may explain volumetric differences as a function of *APOE*-ε4.

3.4.2 Stability of hippocampal volume over one year

These data also converged with that in meta-analyses by Haijma[217] and van Erp[216] in terms of showing that hippocampal volume remains stable and there is no substantial change in early stages of the illness. The contribution of this work was to provide a more precise and direct estimation of when change does NOT occur, as meta-analyses make inferences based on separate samples, not the same group of individuals over time. In addition, data in the present study did not support an antagonistic pleiotropic effect on hippocampal volume over time, in contrast to what was seen with verbal memory in FEP. This suggests that the improvement in verbal memory performance would likely not be mediated by structural changes, but may be mediated by functional mechanisms like increased recruitment of brain areas in healthy *APOE*-ε4 youth demonstrated with fMRI[328,329].

3.4.3 Reduction in volume is not associated with clinical variables

Our results are similar to prior research showing reductions in hippocampal volume are not associated with clinical outcomes or illness severity[217] . However, our data do indicate that factors such as medication or premorbid variables (i.e. duration of untreated psychosis) have an impact on hippocampal volume, and is convergent with recent reports[330]. However, the lack of association of potential confounding variables to hippocampal volume increased the robustness of the claim that the disorder itself and the genotype are mostly driving the effect on reduction of hippocampal volume.

3.4.4 Limitations

These findings draw attention to the causality gap that exists between brain structure and clinical outcomes. Brain morphology is very likely the end result of complex and multifactorial effects at multiple epistemological levels (i.e. molecules, cells, circuits, etc.) and may be very distal to those that have a statistical association. A recent large imaging study attempted to determine the genetic contribution to brain structure[331]. This large study had more than 30,000 brain MRI scans with associated genetic information. The strongest signal corresponded to five novel genetic variants influencing the volume of subcortical structures. The statistical significance was very high and corrected for multiple comparisons. And yet, the genetic variants explained only 0.52% of the variance. This clearly indicates that the current study was severely underpowered to be able to detect a genetic association to a volumetric change. Additional limitations of the present work include the limitation of structural analyses of volumetric measures, including not undertaking other morphological analyses such as shape or hippocampal subfield volumes which could have provided complementary information on morphological abnormalities. Some hippocampal subfield analyses have shown certain specificity between

localization of the abnormalities and particular disorders or genetic variants. In the case of SCZ volume reductions in the cornu ammonis (CA1) and dentate gyrus have been noted[225], although others have reported CA2/3 and CA4/DG[224] deficits. Divergent findings might be explained by more recent data showing that hippocampal abnormalities in SCZ might start in CA1 and expand to other areas in the hippocampus as the illness progresses[227].

3.4.5 Conclusion

To conclude, our data showed that 1) hippocampal volume was reduced in FEP and does not progress over time, and 2) there was a potential additive effect of *APOE*-ε4 on volume reduction in early adulthood, 3) the timing of the morphological changes due to *APOE*-ε4 status may coincide with the window when psychotic symptoms typically emerge in SCZ. Perhaps the current findings point to a common pathophysiological mechanism. In summary, our study emphasizes how our understanding morphogenetics can be fruitful in providing insight into brain development and the pathophysiology of neuropsychiatric disorders.

Chapter 4: Apolipoprotein varepsilon 4 and declarative memory in schizophrenia[o]

4.1 Introduction to chapter 4

4.1.1 Cognitive impairment in schizophrenia and first episode psychosis

In chapter 1, I discussed how the DSM-5 relies on a consensus process to include specific signs and symptoms as diagnostic criteria to build the syndromic diagnosis, and how this process does not include any biomarkers or tests[5]. Cognitive impairment has never been considered a diagnostic criterion in any of the versions of the DSM or the ICD despite the fact that cognitive impairment in SCZ is pervasive, present in early stages and chronic phase of the illness, and during acute episodes as well as in between episodes or periods of remission of psychotic symptoms[23,332,333]. Furthermore, cognitive impairment has consistently been linked and a major factor contributing to functional impairment[21,306].

The importance of cognitive impairment in SCZ is supported by a wealth of data spanning the prodromal phase, early stages of the illness as well as chronic samples. Heinrich and Zakzanis were the first to undertake a meta-analytical approach to summarize and consolidate data available in chronic SCZ patients at the time (late 1990s) in a benchmark work

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[o] A Version of this chapter has been published:

Vila-Rodriguez F, Lang DJ, Baitz H, Gicas K, Thorton AE, Ehmann TS, Smith GN, Barr AM, Torres IJ, Kopala LC, MacEwan GW, Müller DJ, Kennedy JL, Honer WG. Verbal memory improvement in first-episode psychosis *APOE*-ε4 carriers: a pleiotropic effect? Neuropsychiatr Dis Treat. 2017 Dec 8;13:249-257. PMID: 29263671.

in the field (currently accruing more than 2,500 citations)[23]. The meta-analysis included a total of 204 studies investigating cognitive impairment encompassing all the main neuropsychological domains. The sample derived from all the studies consisted of a total of 7,420 SCZ patients and 5,865 healthy controls. The SCZ sample was composed of 82% males, the mean age of patients was 34 years old, the mean age of onset 22.2 years old, the mean years of education was 12 years. Clinically, these patients had a mean number of hospital admissions of 3.9, their mean length of illness was 12.7 years, and the mean CPZ daily equivalent dose was 582.1 mg/d. Meta-analysis showed that cognitive deficits were of moderate or large effect size across all the cognitive domains ranging from -1.41 to -0.41; the largest effect size corresponding to deficits in verbal memory (-1.41), and 78% of patients were below the median performance of CTRL. This work provided a robust benchmark to gauge cognitive deficits in other phases of the illness, and also quantified the magnitude of the cognitive impairment in terms of its pervasiveness in the disorder as well as the severity of it.

Mesholam-Gately, Giuliano, and colleagues leveraged the work by Heinrich and Zakzanis 10 years later to undertake a meta-analysis with similar structure in FEP[332]. In doing so, they set the stage to better understand the course of cognitive impairment in SCZ. The meta-analysis included 47 studies corresponding to 43 separate samples totaling 2,204 FEP and 2,775 age- and sex-matched healthy volunteers. The FEP was comprised of 65% men, mean age was 25.5 years old with a mean number of years of education of 11.8 years. Clinically, the mean number of admissions was 1 and 62.6% were on antipsychotics. Unfortunately, the length of the illness or CPZ daily equivalents was not retrieved as it was in the chronic SCZ meta-analysis. Nonetheless, the FEP meta-analysis sample was clearly comprised of a younger population in much earlier stage of the illness and with a significant proportion of patients who were

antipsychotic naïve and had accrued a lower lifetime antipsychotic dose accrued. As in chronic SCZ, FEP subjects had cognitive impairment across neuropsychological domains of large to moderate effect size ranging from -1.20 to -0.64. Similar to chronic SCZ, in FEP immediate verbal memory domain was the neurocognitive domain with the largest effect size (-1.20). Similarities in pervasiveness of neurocognitive impairment and severity between early stages and chronic SCZ can be fully appreciated in Table 4.1.

	Chronic SCZ Effect Size (95% CI)	FEP Effect Size (95% CI)
Immediate Verbal Memory	-1.41 (-1.20 to -1.62)	-1.20 (-1.05 to -1.35)
Delayed Verbal Memory and Learning Strategies	-0.9 (-0.44 to -1.36)	-0.85 (-0.71 to -0.99)
Nonverbal Memory	-0.74 (-0.30 to -1.78)	-0.91 (-0.79 to -1.03)
Executive Functioning	-0.88 (-0.76 to -1.00)	-0.83 (-0.72 to -0.95)
Attention-Vigilance	-1.16 (-0.90 to -1.42)	-0.71 (-0.55 to -0.87)
Motor Skills	-0.86 (-0.55 to -1.17)	-0.64 (-0.52 to -0.77)

Note: While all the neurocognitive domains in Mesholam-Gately's meta-analysis are based on composite scores drawn from multiple tests, in Heinrichs and Zakzanis "Executive Functioning" corresponds to only Wisconsin Card Sorting Test, "Attention-Vigilance" to Continuous Performance Tests, and "Motor Skills" to Unilateral Motor Skills.

Table 4.1 Comparative neurocognitive deficits in chronic schizophrenia and first-episode psychosis. Table based on data from meta-analyses by Heinrichs and Zakzanis[23] and Mesholam-Gately at al[332].

These data show that from early stages of the illness when psychotic symptoms initially emerge to the subsequent 15 years, there is no marked progression of neurocognitive deficits. This prompts the next logical question in this backwards sequence, are there cognitive deficits before the onset of psychotic symptoms? What is the neurocognitive impairment during the prodromal phase of the illness?

The meta-analysis by Woodberry and colleagues[289] offers important insights to address those questions. This meta-analysis included a total of 18 studies including more than 2,500 individuals for whom premorbid standardized psychometric test allowed estimation of general IQ (majority achievement tests). Although the heterogeneity of methods and data reported in studies was significant, and overall estimated IQ impairment of -0.54 (Cohen d effect size) was reported. This data point allowed for a comparison with the overall estimated IQ in meta-analysis in FEP and chronic SCZ and provided evidence that a significant global cognitive impairment is already present in the premorbid phases of the illness and that the most significant drop in estimated IQ would occur between the premorbid phase and the onset of overt psychosis (see Figure 4.1).

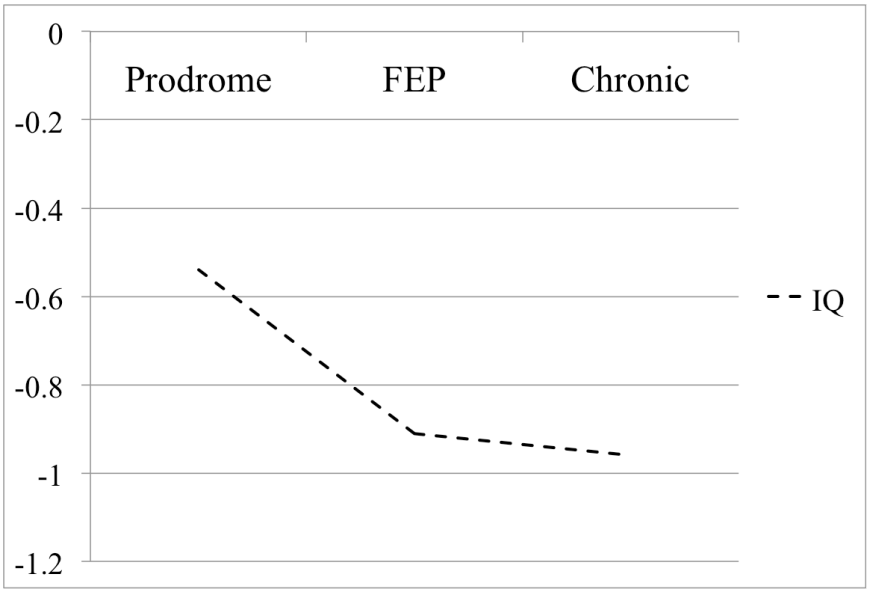


Figure 4.1 Effect size of estimated IQ in the premorbid stage, compared to early and chronic stages in schizophrenia (plot adapted from Meshola-Gately et al.)

Taken together, these three meta-analyses provide solid evidence of the pervasiveness and severity of neurocognitive impairment in SCZ as well as the course and natural history of these neurocognitive deficits. Whether there is any change (particularly progression) over time after the onset of psychosis has remained a topic of controversy due to divergent findings in studies assessing change over time in cognition, including some studies reporting an improvement in neurocognition associated with neuroleptic treatment[334,335]. The latest meta-analysis conducted on those studies assessing neurocognition longitudinally by Bozikas and colleagues from 2011 included 26 studies comprising a total of 1,527 patients with SCZ spectrum disorders (not all studies included a control group) who were assessed for more than one year apart[336]. The results indicated that neurocognitive impairments remain stable for up to 10 years, with the only exception of mild progressive worsening of verbal memory impairment.

Subsequently, large studies following up FEP patients for 10 years strengthened the claim of neurocognitive impairment stability over the course of the first 10 years after the onset of psychosis[337].

The consistency and quality of evidence supporting the presence of neurocognitive impairments in SCZ confirms the reliability of neurocognitive deficits as a trait marker of SCZ. The etiopathogenesis of such neurocognitive deficits remains to be elucidated. Studies showing the presence of moderate effect size in non-psychotic twins[31], and a recent meta-analysis showing the existence of moderate effect size of neurocognitive impairment in non-psychotic relative of SCZ patients[338] implicate a genetic factor underlying the etiology of these deficits.

4.1.2 *APOE*, declarative memory and psychosis

One potential polymorphism of interest in SCZ-associated verbal memory impairment is the Apolipoprotein E gene (*APOE*). The Apolipoprotein E gene presents in three main alleles in humans: *APOE* varepsilon 3 (*APOE*-ε3), 79%; *APOE* varepsilon 4 (*APOE*-ε4), 14%; and *APOE* varepsilon 2 (*APOE*-ε2), 8%[339]. *APOE* codifies for a protein, Apolipoprotein E, that has a critical role in synaptogenesis, neurite outgrowth, and membrane repair and maintenance[57,60,246,249]. In addition, *APOE*-ε4 is the main genetic risk factor for late-onset Alzheimer's disease where cognitive impairment is the core manifestation of the disorder[310], and has been associated with verbal memory impairment in this condition[340]. Furthermore, *APOE*-ε4 carrier status has been associated with the severity of neuropathological findings such as hippocampal neurofibrillary tangles, neurite plaque density, and severity of dementia in brains from SCZ patients[125], and some studies report earlier onset of SCZ[105], association to positive symptoms[76], or to negative symptoms[100].

4.1.3 *APOE* and declarative memory

The relationship between *APOE*-ε4, SCZ, and verbal memory is complex. First, although early studies and meta-analysis showed increased *APOE*-ε4 carriers status associated with SCZ[76,134], this finding was not universally replicated[91,102,133] and the most recent meta-analysis by Gonzalez-Castro and colleagues did not replicate earlier meta-analysis results[133]. Second, recent research investigating the course of cognitive function and genetic risk in psychiatric disorders showed that genetic associated cognitive impairment was worse in SCZ only in older age, but not in young populations, suggesting a pleiotropic effect[341].

4.1.4 Antagonistic pleiotropic effect

George C. Williams, an evolutionary biologist, postulated the antagonistic pleiotropic theory in his seminal work in 1957[342]. He articulated the concept that certain genes may increase the chances of survival or reproduction rate (i.e. fitness), at the expense later on of accelerating senescence. Antagonistic pleiotropy's simplest and plain expression can be articulated by stating that a gene that may be good early in life ("good gene" in youth) may be bad later on ("bad gene" in the elderly). *APOE*-ε4 is hypothesized to be one of such genes[343], and there is converging evidence supporting this hypothesis. First, *APOE*-ε4 seems to confer slight increased fertility rates in humans[149,150] (increase fitness; good gene). Second, in youth *APOE*-ε4 seem to be associated with better performance in verbal memory, verbal fluency, higher IQ, or better developmental indices in infants[328,344–346]. A potential mechanism would appear to be a different pattern of brain activity as shown by fMRI[329]. This functional difference is lost by middle age[347] and later on *APOE*-ε4 is the main genetic risk factor for late-onset AD[64,66].

4.1.5 Aims and hypotheses

Aim 1. To investigate the effect of *APOE*-ε4 on verbal memory deficits in first-episode psychosis.

Aim 2. To investigate the effect of *APOE*-ε4 on verbal memory deficits over time in first-episode psychosis.

Hypothesis 1.1. *APOE*-ε4 will be associated with verbal memory deficits related to encoding impairment.

Hypothesis 1.2. *APOE*-ε4 will be associated with an antagonistic pleiotropic effect on verbal memory.

Hypothesis 2. *APOE*-ε4 will be associated with an antagonistic pleiotropic effect over time.

4.2 Material and methods

4.2.1 Methods

The research protocol was approved by the University of British Columbia and Vancouver Coastal Health Authority Clinical Research Ethics Boards, and was in accordance with Tri-Council policy. Written informed consent was obtained from all participants after the procedures had been fully explained. Participants were recruited from a longitudinal study from an Early Psychosis Intervention program serving a catchment area population of 640,000 in the Vancouver area. All patients referred to the program were approached to enter the study. Participants had less than 8 weeks of total lifetime exposure to antipsychotic treatment at the time of cognitive assessment.

Inclusion criteria for patients for this study were presentation of DSM-IV defined psychotic disorders, and fluency in English. Exclusion criteria were i) history of significant head

injury defined by loss of consciousness greater than 5 minutes, ii) history of neurodevelopmental disorder or central nervous system infection, iii) history of inhalant intoxication, iv) inability to provide full written consent, and v) IQ less than 75. Inclusion and exclusion criteria were similar for healthy volunteers, with additional exclusion for history of major psychiatric disorder.

Diagnoses were based on the Structured Clinical Interview for DSM-IV, a review of clinical case notes, and interviews with one or more family members. This information was presented at a case conference, and two psychiatrists and a clinical psychologist made a consensus diagnosis. A detailed review of past and current substance use was obtained at baseline and follow-up. The Positive and Negative Syndrome Scale (PANSS;[313]) estimated psychiatric symptom severity at baseline and follow-up. A five-factor solution was computed at baseline and follow up based on a meta-analysis of FES[314]. CPZ equivalents were computed according to Woods and colleagues[315]. Level of functioning was indexed at baseline using the Social and Occupational Functioning Assessment Scale (SOFAS;[273],clinical global impression (CGI;[316]) and the Role Functioning Scale (RFS;[317]).

4.2.1.1 Participants

The sample included 86 FEP subjects and 39 healthy volunteers (control [CTRL]). The FEP group consisted of SCZ patients (n = 44), schizoaffective patients (n = 13), affective psychosis patients (n = 17), and other psychosis patients (n = 12). FEP and CTRL groups were similar in mean age, sex, ethnicity distribution, and prevalence of *APOE*-ε4 carriers. The FEP group had lower estimated pre-morbid IQ as well as current IQ. Similarly, the FEP group had statistically significant fewer years of education (Table 4.2).

	FEP (n = 86)	CTRL (n = 39)
Male:Female (%Male)	59:27 (68.6%)	25:14 (64.1%)
Age, yrs; mean (SD)	20.4 (4.2)	20.8 (3.7)
Ethnicity; Cau:Asi:Other (% Cau)	60:13:13 (69.8%)	26:7:6 (66.7%)
APOE-ε4 carriers*; n (%)	29 (33.7%)	8 (20.5%)
Education; yrs; mean (SD)	11.6 (1.7)	13.4 (2.5) $^{+}$
NAART FSIQ; mean (SD)	100.4 (8.0)	107.5 (6.7) $^{+}$
K-BIT IQ; mean (SD)	94.7 (11.7)	108.5 (9.9) $^{+}$

*One *APOE2*/4 was excluded; $^{+}$ Statistical significance $p < .001$

Abbreviations: FEP First Episode Psychosis; CTRL Control; yrs years; S single; P Partner; Cau Caucasian; Asi Asian; NAART FSIQ North American Reading Test Full Scale IQ; K-Bit IQ Kauffman Brief Intelligence Test IQ.

Table 4.2 Demographic characteristics and premorbid and current intelligence quotient (IQ) of the sample

A summary of demographic and clinical characteristics comparing the FEP *APOE*-ε4 carriers group (FEP-*APOE*-ε4+) vs. the FEP non-*APOE*-ε4 carrier group (FEP-*APOE*-ε4−) at baseline is presented in Table 4.3, including medications at time of neuropsychological assessment and number of patients who were antipsychotic-naïve at the time of

neuropsychological assessment. In summary, both groups were similar in mean age, mean years of education and sex distribution.

FEP sample only	**Non-*APOE*-ε4 (n = 57)**	***APOE*-ε4 (n = 29)**
Male: Female (% Male)	40:17 (70.2%)	19:10 (65%)
Age in years; mean (SD)	20.6 (4.4)	20.1 (3.8)
Ethnicity; Cau:Asi:Other (%Cau)	39:9:9 (68.4%)	21:4:4 (72.4%)
Education in years; mean (SD)	11.5 (1.6)	11.6 (1.9)
NAART; mean (SD)	101.0 (7.8)	99.4 (8.5)
K-BIT IQ ; mean (SD)	95.6 (10.7)	92.8 (13.4)
Diagnoses, SCZ:SCZ_A:AfP:other (% SCZ)	29:9:11:8 (51%)	15:4:6:4 (52%)
PANSS Scores; mean (SD) Positive Negative General Total	 19.3 (6.5) 18.1 (6.9) 41.1 (9.3) 78.5 (19.5)	 19.1 (5.1) 19.1 (5.1) 41.8 (7.3) 80.0 (15.8)
CGI; mean (SD)	4.2 (0.9)	4.4 (0.7)
Onset of symptoms: Age First Decline, mean (SD) Age First Psychosis, mean (SD) DUP (mo); mean (SD)	 17.7 (4.6) 19.0 (4.7) 22.3 (27.2)	 17.6 (4.5) 19.0 (4.7) 17.2 (22.2)
Medications: Ris/Ola/other/missing CPZ; mean(SD) AP naïve; n(%)	 17/16/3/8 210.6 (196.5) 13 (26%)	 14/6/0/1 140.7 (107.9) 8 (29%)

Abbreviations: NAART: North American Reading Test; K-Bit IQ: Kauffman Brief Intelligence Test IQ; SCZ: Schizophrenia; SCZ-A: Schizoaffective; AfP: Affective psychosis; DUP: Duration of untreated psychosis; Ris: Risperidone; Ola: Olanzapine; AP: Antipsychotic; PANSS: Positive and Negative Syndrome Scale; CGI Clinical Global Impression;

Table 4.3 Clinical characteristics of first episode psychosis patients, non-*APOE*-ε4 carriers vs. *APOE*-ε4 carriers

In addition, both groups had similar premorbid and current estimated IQ. The two groups were similar with respect to symptom severity, level of functioning, and medication status.

4.2.1.2 *APOE* genotyping

Genotyping of the *APOE* rs7412 and rs429358 polymorphisms was performed using standard ABI (Applied Biosystems Inc., Foster City, CA, USA) SNP Genotyping Assays. Genotype calls were performed manually. Laboratory personnel blind to demographic and phenotypic information independently verified the results.

To ensure quality control, 10% of the samples were randomly re-genotyped with a 100% concordance rate.

4.2.1.3 Neuropsychological assessment

Trained research assistants under the supervision of a clinical neuropsychologist completed neuropsychological testing. Cognition was evaluated at the baseline and 9- to 12-month time points. Premorbid intellectual functioning was estimated at baseline with the North American Adult Reading Test (NAART;[320]). Current IQ was estimated at baseline with the Kaufman Brief Intelligence Test (K-BIT; [321]). Verbal memory was assessed using the California Verbal Learning Test 2nd Edition (CVLT-II-[322]).

4.2.1.3.1 Description of neuropsychological testing

North American Adult Reading Test[320] (NAART)

The NAART is a test derived from the classic National Adult Reading Test (NART[348]) for North American population. A core feature of the test relies on testing the correct pronunciation of a list of words and this notably varies between English in the UK, where the original NART was developed, and North-American English[320]. The psychometric properties of the test have been tested and shown to have a good correlation with general IQ, although this

changes when the IQ deviates significantly from the normative population[320,349–351]. The rationale behind using the NAART as an estimate of premorbid general IQ is based on data showing that word pronunciation is crystallized cognitive ability meaning that it is consolidated very early during development and is spared from deterioration until a morbid process is severely advanced. The NAART is considered a "hold" test (i.e. a test that measures crystallized abilities that are relatively insensitive to brain damage) as opposed to "non-hold" tests that ascertain cognitive abilities that are susceptible to worsening due to brain damage. Because the usefulness of the NAART to estimate premorbid IQ depends on prior familiarity with the words, the NAART is a test that correlates with prior level of education. This should be considered during interpretation of scores and data analyses.

Kaufman Brief Intelligence Test[321] (K-BIT)

The K-BIT was designed as a brief test to measure both verbal (crystallized) and non-verbal (fluid) intelligence as well as a global score estimate of general IQ. The K-BIT has demonstrated a good correlation with more comprehensive IQ test such as the Wechsler IQ test. The correlation tend to be higher for the verbal IQ more than the non-verbal component[352–354].

The matrices subtest is composed of several types of items involving visual stimuli, both meaningful (people and objects) and abstract (designs and symbols). The stimuli have a common denominator and the subject is given a multiple choice to point or say its letter.

California Verbal Learning Test[322]

The California Verbal Learning Test (CVLT) is a neuropsychological test developed to ascertain multiple process involved in verbal memory rather than giving a single global score [355]. The CVLT was inspired by the Rey Auditory Verbal Learning Test (RAVLT [356]). It differs from the RAVLT by using categorized word lists, adding a cued recall task, and testing recognition by

a word list rather than a story. The manual describes the following domains: i) learning, ii) serial position effects (primacy, recency), iii) Semantic Organization (semantic clustering, cued recall), iv) Retention (delayed recall), v) Recognition, vi) intrusions and perseverations, vii) interference.

The CVLT was developed with the prime objective of not only ascertaining general memory capacity but foremost to ascertain how subjects learn (see Figure 4.2 for a visual summary of test). As an example, the words are drawn from four semantic categories (tools, fruits, clothing, spices and herbs), with no consecutive words from the same category. Therefore, if subjects tend to put together words each of the categories they are presumably using a semantic organization strategy to aid their recall.

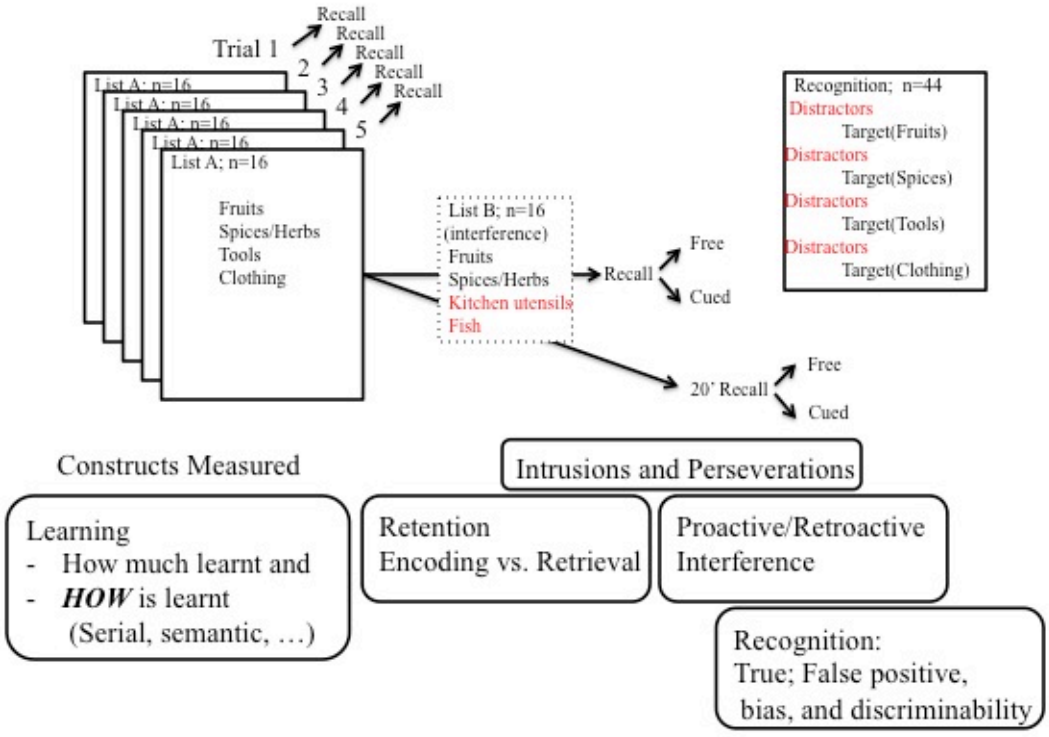


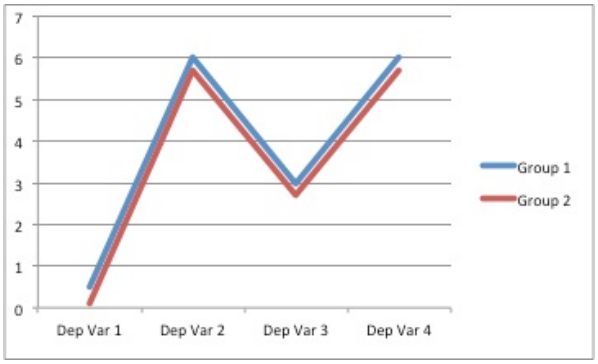
Figure 4.2 California verbal learning test

4.2.1.4 Statistical analyses

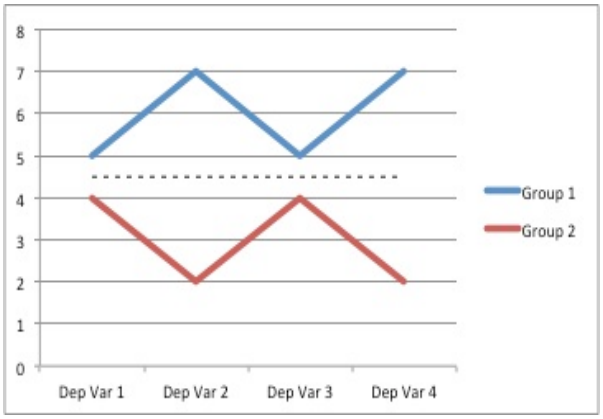
Statistical analyses were performed with SPSS (version 24.0; IBM corp). Data Exploratory Analysis was used to ascertain the characteristics of the distribution of the variables, including testing for normality.

Profile analysis is an extension of repeated measures MANCOVA where multiple independent variables (or predictor variables) are on the same scale (commensurate). Similar to repeated measures MANCOVA there are three principal contrasts namely a between-group, a within-subjects, and a group-by-subjects interaction. However, in profile analysis there are no true repeated measures. Instead, dependent variables can be tested for a within- effect as they are on the same scale (i.e. the same way that would happen for a repeated measure). The question that profile analysis allows asking is "Do groups have similar profiles on **a set of dependent variables**?" The main analysis is not of a single dependent variable, but rather a group of variables[357]. The between-subjects contrast in profile analyses tests whether the set of independent variables is different between the levels of the dependent variable, or whether there is a main effect (Figure 4.3 A). The within-subjects question in profile analysis is termed "test of flatness". Again, the repeated measures in profile analysis are not standard repeated measures, but dependent variables on a commensurate scale. In this case the within-subjects becomes a "within-dependent variables" contrast. The contrast tests whether the dependent variables elicit the same average response (Figure 4.3 B). Third, the "test of parallelism" in profile analysis is the interaction between the dependent variable and independent variables, or whether profiles are coincident geometrically. An interaction occurs when the profiles are not parallel (Figure 4.3 C).

A.



B.



C.

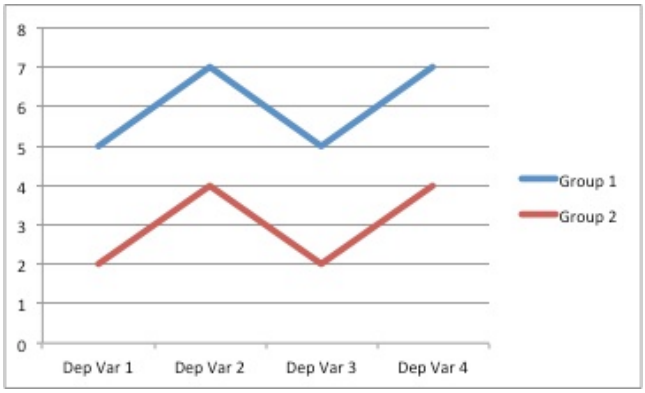


Figure 4.3 Visual representation of hypotheses tested in profile analysis.

A. Plot representing the between-group main effect null hypothesis in profile analysis; B. Plot representing the flatness null hypothesis; C. Plot representing the parallelism null hypothesis.

Six primary CVLT-II standard scores were treated as within-subjects variables analogous to repeated measures (i.e., standardized CVLT-II scores on Trial 1, Trial 5, Short Delay Free Recall, Short Delay Cued Recall, Long Delay Free Recall and Long Delay Cued Recall[322]) and group (FEP and CTRL) was the between-subjects variable. Profile analysis tested for separation (main effect), parallelism, and flatness of the plotted patient and control CVLT-II-score profiles[357]. Analysis of variance was used to test the null hypothesis of equal means of verbal memory across four types of medication (dependent variable CVLT-II Trials 1-5, independent variable type of medication at baseline). CVLT-II standardized scores were used.

SPSS MIXED procedure was used to perform a mixed-model repeated measures (MMRM) analysis of the relationship of verbal memory performance as indexed by the standardized CVLT-II Trials 1-5, diagnosis and *APOE*-ε4 status over time. We chose CVLT 1-5 as it represents the most reliable summary measure of immediate verbal memory[322]. For fixed effects, we entered diagnosis (FEP vs. CTRL) and *APOE*-ε4 status (*APOE*-ε4 carriers vs. non-*APOE*-ε4 carriers) as between-subjects, and time (baseline vs. follow up) as within-subjects factor with the triple interaction term included in the model. Age was entered as a covariate. An unstructured covariance matrix was assumed. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality.

Post hoc power calculations were computed using G*Power software[358].

4.3 Results

4.3.1 Profile analysis of CVLT scores in FEP vs. CTRL

Profile analysis was used (i.e. repeated measures MANCOVA). First, the assumption for sphericity was violated (Mauchly's W = 0.28; Approx $\chi^2 = 228.4$; $p < .001$). Subsequently, the Greenhouse-Geisser statistic was used. There was a main effect of diagnosis as CVLT scores were significantly lower in FEP ($F (1, 122) = 50.8$, $p < .00001$, $h^2 = .29$). The test for flatness (i.e. within-subtests) indicated that performance was not even across CVLT subtests ($F (5, 118) = 2.42$, $p = .04$, $h^2 = .09$). However, follow-up comparisons for each pairwise difference were non-significant after the Bonferroni correction for multiple comparisons. The test for parallelism indicated there was no diagnosis by subtests interaction as both FEP and CTRL had coincident profiles $F (5, 118) = 1.16$, $p = .30$, $h^2 = .05$). Profiles are displayed in Figure 4.4.

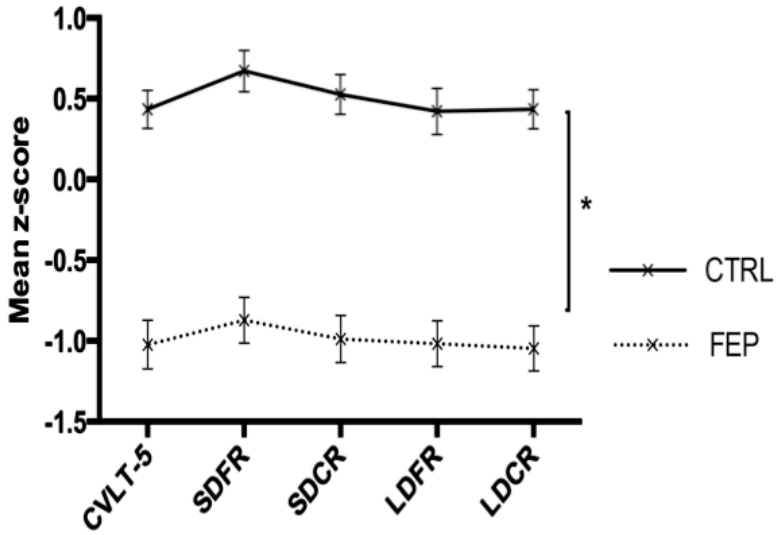


Figure 4.4 Profiles of verbal memory performance in FEP vs. CTRL

4.3.2 Effect of *APOE*-ε4 status on verbal memory profile in controls at baseline

Profile analysis was used (i.e. repeated measures MANCOVA), (see Figure 4.5). First, the assumption for sphericity was violated (Mauchly's W = 0.55; Approx $\chi^2 = 20.4$; p = .016). Subsequently, the Greenhouse-Geisser statistic was used. There was no main effect of genotype as *APOE*-ε4 carriers and non- *APOE*-ε4 carriers performed similarly on verbal memory subtests (F (1, 36) = 4.7, p = .16, $h^2 = .054$). The test for flatness (i.e. within-subtests) indicated that that scores across CVLT domains were similar (F (3.3, 118.6) = 1.6, p = .18, $h^2 = .044$). The test for parallelism indicated there was no genotype by verbal memory subtests interaction as *APOE*-ε4 carriers and non-*APOE*-ε4 carriers had coincident profiles F (3.3, 118.6) = 0.41, p = .76, $h^2 = .01$).

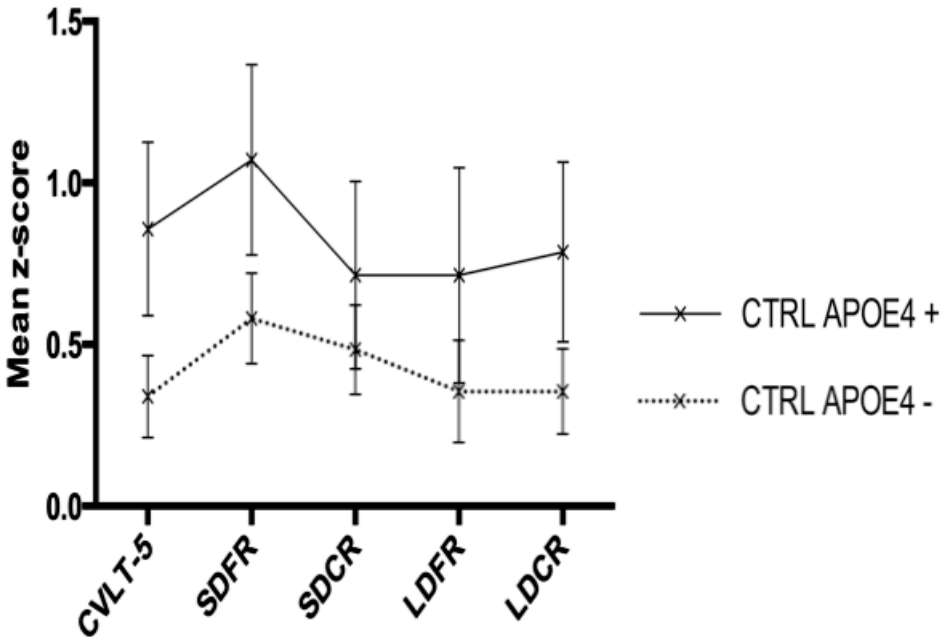


Figure 4.5 Profile analysis of verbal memory performance in controls: comparing *APOE*-ε4 carriers vs. non-*APOE*-ε4

4.3.3 Effect of *APOE*-ε4 status on verbal memory profile in FEP at baseline

Profile analysis was used (i.e. repeated measures MANCOVA), (see Figure 4.6). First, the sphericity assumption was violated (Mauchly's W = 0.38; Approx $\chi^2 = 78.69$; $p < .001$). Subsequently, the Greenhouse-Geisser statistic was used. There was no main effect of *APOE*-ε4 status on verbal memory performance as *APOE*-ε4 carriers and non-*APOE*-ε4 carriers performed similarly ($F (1, 84) = 0.03$, $p = .87$, $h^2 < .001$). The test for flatness (i.e. within-subtests) indicated that verbal memory performance was similar across the CVLT subtests ($F (3.8, 320.8) = 1.08$, $p = .36$, $h^2 = .02$). The test for parallelism indicated there was no genotype by verbal memory subtests interaction as *APOE*-ε4 carriers and non-*APOE*-ε4 carriers had coincident profiles $F (3.8, 320.8) = 0.408$, $p = .692$, $h^2 = .01$).

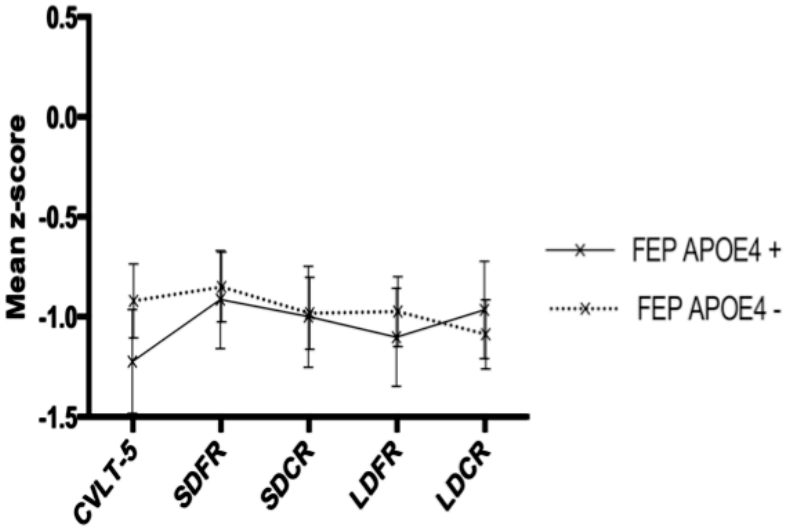


Figure 4.6 Profile analysis of verbal memory performance in FEP at baseline: comparing *APOE*-ε4 carriers vs. non-*APOE*-ε4

4.3.4 Change in verbal memory performance over time

Mixed-model repeated measures analyses revealed a main effect of group on CVLT-II Trials 1-5 (FEP = 43.30 vs. CTRL = 58.25; F (1, 119.7) = 42.97; p < .001), but no main effect for APOE-ε4 status (*APOE*-ε4 carriers = 52.27 vs. *APOE*-ε4 carriers non-carriers = 49.28; F (1, 119.7) = 1.72; p = .19) or Time (Baseline = 51.05 vs. Follow up = 50.49; F (1, 109.2) = 0.17; p = .68). However, MMRM analyses revealed an interaction of *APOE*-ε4 by Group by Time (F (4, 116.2) = 2.73, p = .033). I followed up on this interaction by plotting the marginal means in memory performance across time by subgroup as shown in Figure 4.7[359]. Visual inspection of this plot clearly reveals the effect is mostly driven by change over time in FEP *APOE*-ε4 carriers. Informed by this visual analytic technique I followed up with *post hoc* analyses in FEP *APOE*-ε4 carriers using a paired t-test. Results showed a significant difference in verbal memory performance at follow-up in FEP-*APOE*-ε4 carriers (Baseline = 42.92; Follow up = 48.36; t (24) = -2.40, p = .025). There was no difference in the lapse of time between the two neuropsychological sessions between diagnostic groups or between *APOE*-ε4 carriers and non-carriers.

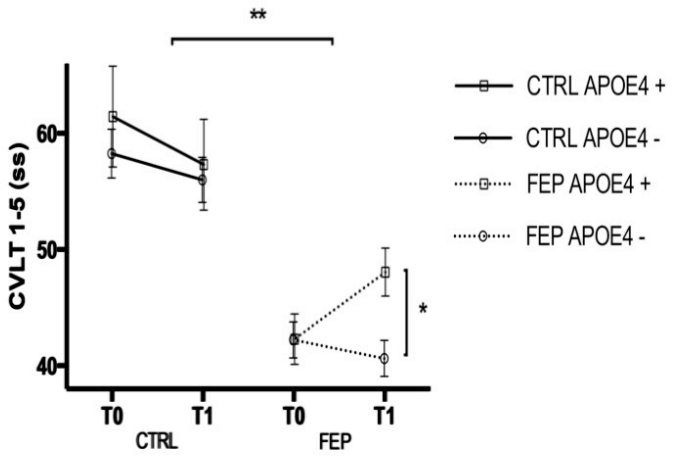


Figure 4.7 CVLT 1-5 change over time in FEP and CTRL

Post-hoc power calculation for main effect of *APOE*-ε4 (carriers vs. non-carriers) was estimated with an F test, repeated measures, between factors. The input provided to G*Power consisted of effect size f =1.358594; α error probability = 0.05; total sample size = 137; number of groups = 2; number of measurements = 2; correlation among repeated measures = 0.5. G*Power ouput was a noncentrality parameter λ = 337.1; critical F = 3.9; numerator df = 1; denominator df = 135, and a Power (1-β error probability) estimate = 1.0000000. *Post-hoc* power calculation for main effect of diagnosis (FEP vs. control) was estimated with an F test, repeated measures, between factors. The input provided to G*Power consisted of effect size f =6.949647; α error probability = 0.05; total sample size = 137; number of groups = 2; number of measurements = 2; correlation among repeated measures = 0.5. G*Power ouput was a Noncentrality parameter λ = 8822.4; Critical F = 3.9; numerator df = 1; denominator df = 135; Power (1-β error probability)=1.0000000. *Post-hoc* power calculation for an interaction of group by *APOE*-ε4 status by time was estimated with an F test, repeated measures, between-within interaction. The input provided to G*Power consisted of effect size f = 0.25; α error probability = 0.05; total sample size = 137; number of groups = 2; number of measurements = 2; correlation among repeated measures = 0.5; Nonsphericity correction ε = 1. G*Power ouput was a Noncentrality parameter λ= 34.2; Critical F = 3.9112667; numerator df = 1; denominator df =135; Power (1-β error probability) = 0.9999410.

4.3.5 Effect of medication on verbal memory performance and *APOE*-ε4

Medications at the time of cognitive testing were similar in FEP-*APOE*-ε4 carriers vs. FEP-*APOE*-ε4 non-carriers (Table 4.3). There was no significant correlation of total accumulated dose of antipsychotic at baseline in CPZ equivalents and verbal memory performance at baseline (CPZ dose vs. CVLT-II Trials 1-5 r = .02, p = .88), nor was the mean CVTL-II Trials 1-5 score

statistically different as a function of treatment (one-way ANOVA, Olanzapine = 44.23 vs. Risperidone = 43.11 vs. naïve = 41.38; F (2,73) = 0.323; p = .725). The percentage change of CPZ dose from baseline to follow up was not statistically correlated with percent change in verbal memory performance from baseline to follow up (percent change CPZ dose vs. percent change CVLT-II Trials 1-5 r = -.01, p = .94). *APOE*-ε4 status was not correlated with the percent change in CPZ dose (r = .06, p = .7). Also, prior work in this sample showed no significant correlation of anticholinergic load and performance on CVLT- II Trials 1-5 at follow up [360].

4.3.6 Verbal memory performance and *APOE*-ε4 effects on clinical outcomes

There were no significant differences between FEP-*APOE*-ε4 carriers and non-carriers in total or subscale PANSS scores, clinical global impression scores or level of functioning on the role functioning scale at baseline (Table 4.3). All premorbid clinical measures aspects such as mean age of first decline, mean age of first psychosis or mean duration of untreated psychosis were similar in both groups (Table 4.3).

The percent change in verbal memory performance between baseline and follow-up was not correlated with any percent change in PANSS, GAF, CGI, or RFS (data not shown). *APOE*-ε4 status was not correlated with any percent change in PANSS, GAF, CGI, or RFS (data not shown), but *APOE*-ε4 status was associated with verbal memory performance percent change (r = .32, p = .008).

Better verbal memory performance at follow up was correlated with less psychopathology (CVLT-II Trials 1-5 vs. PANSS total score r = -.24; p = .047) as well as less global severity (CVLT 1-5 vs. CGI r = -.3; p = .013). There was a trend for better verbal memory at follow-up being correlated with better social functioning (CVLT-II Trials 1-5 vs. RFS score r = .23, p = .058). *Post hoc* correlations tests for the subscales of PANSS and RFS showed that

better verbal memory performance at follow up was specifically associated with lower psychopathology scores on the negative subscale of PANSS (r = -.35; p = .003) and better extended social network relationships subscale of RFS (r = .25; p = .037).

4.4 Discussion

4.4.1 Large and generalized verbal memory impairment in FEP

These data replicated the finding that verbal memory is impaired in FES, and the magnitude of the impairment is very large. Computing the effect size demonstrated that impairment was present across all the subdomains of verbal memory and this effect was larger than 1 in all the CVLT subdomains (Figure 4.8). In comparison to previously reported data in the Mesholam-Gately FEP meta-analysis[332], we observed a similar effect size albeit slightly more pronounced in our current work (Figure 4.8). This difference in magnitude might be in part due to the difference in the total years of education between patients and CTRL in our study that is not present in the FEP meta-analysis. In the Mesholam-Gately sample, the mean years of education for FEP vs. CTRL in the FEP meta-analysis were 11.8 vs. 12.5 (0.7 years of difference), whereas in the present sample they were 11.5 vs. 13.4 (1.9 years of difference; Table 4.3 above). As verbal memory is a measure that moderately correlates positively with years of education the effect size in our sample might also reflect in part a higher level of education in CTRL.

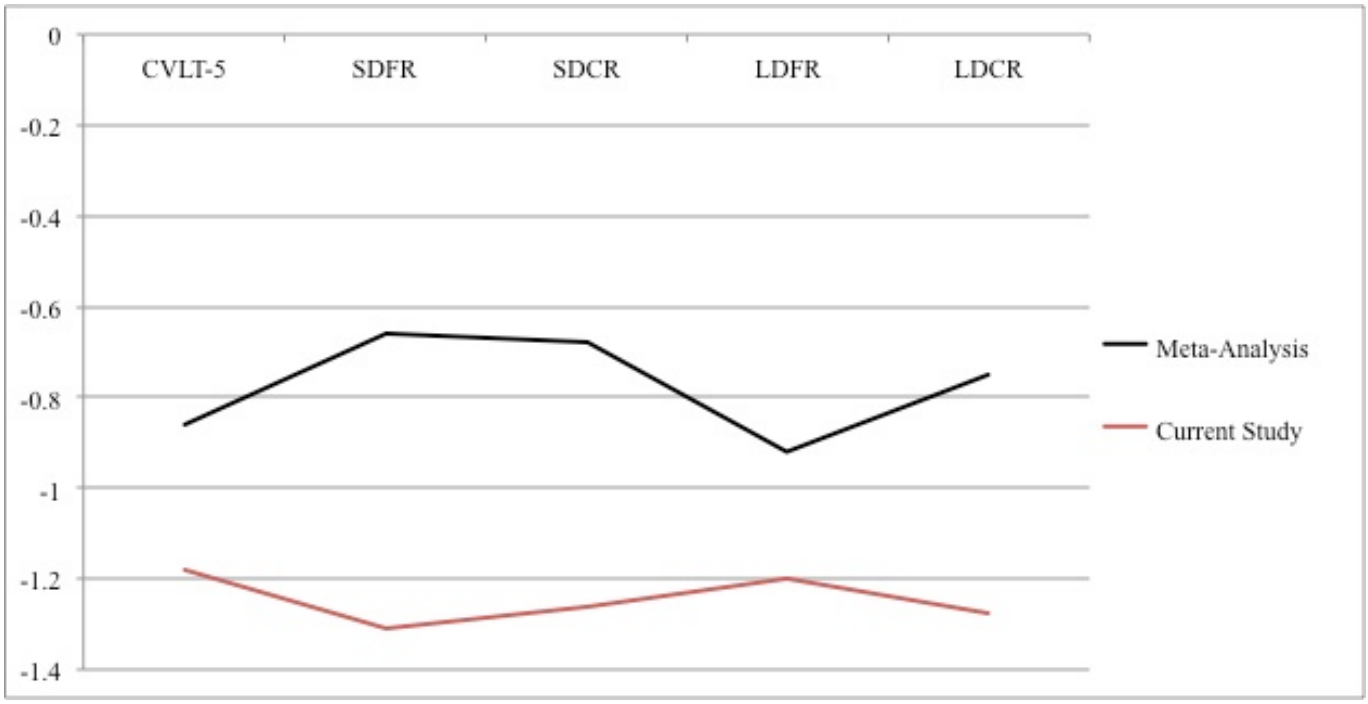


Figure 4.8 Comparison of effect sizes across CVLT subdomains between Meshola-Gately's FEP meta-analysis[332] and current study

The solid black line represents the effect sizes from the meta-analysis and the solid red line represents the effect sizes on the same CVLT subdomains in the current work.

The test of flatness (i.e. within-subtests) indicated that performance was similar across the CVLT subtests in FEP, and this would indicate that the impact on verbal memory is generalized rather than focalized on a particular domain (Figure 4.8). This pattern of similar impairment across verbal memory subtests would argue against a focalized abnormality underlying verbal memory impairment in FEP. Our data are convergent with studies using CVLT in SCZ and demonstrating that verbal memory impairment encompasses impaired performance on learning, recall, and recognition measures. This pattern would be indicative of normal storage[361–363] (Figure 4.9). In addition, our data supports prior reports demonstrating that there is a similar deficit in both retrieval and encoding processes[364].

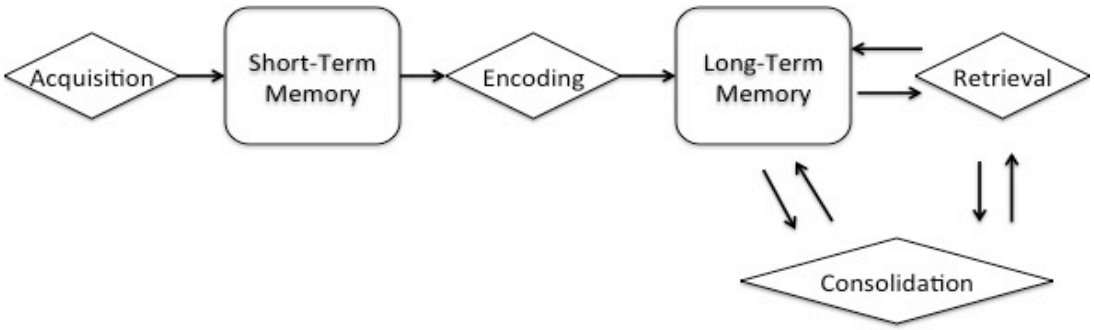


Figure 4.9 Learning and memory systems and processes[365,366]

Information is acquired through our senses and initially processed into a short-term memory system. Information can be retained in this initial 'storage' system for a short span of time unless is further processed or encoded in a way that can be stored in a more long-term memory system. Information stored in this long-term system can then be retrieved and over time the information may undergo a process of consolidation as well. The consolidation of information would be closely linked to the process of retrieval and both in turn would contribute to further consolidate information in the long-term storage system.

4.4.2 The *APOE*-ε4 effect of on verbal memory profile

Data from patients and CTRL did not reveal different profiles as a function of *APOE*-ε4 status. However, visual inspection of the profiles in Figure 4.5 above for CTRL shows that *APOE*-ε4 carriers consistently outperform their non-*APOE*-ε4 carrier counterparts. However, it is important to note that the study by Mondadori and colleagues showing better verbal memory performance in young (mean age 22 years old) healthy *APOE*-ε4 carriers recruited a total of 340 subjects and of those 86 were *APOE*-ε4 carriers. Lack of statistical significance in our study may be due to insufficient statistical power when the sample broke down into 31 non-*APOE*-ε4 carriers and only 7 *APOE*-ε4 carriers. The plot for the FEP group did not reveal any pattern consistent with an advantage of carrying the *APOE*-ε4 allele.

4.4.3 A pleiotropic effect of *APOE*-ε4 over time in FEP?

As shown in Figure 4.7, the data indicated an interaction effect between carrying the *APOE*-ε4 allele, FEP and *improvement* in verbal memory over time. This finding is counterintuitive as the *APOE*-ε4 allele has been shown to be detrimental or a risk factor in a wide array of neuropsychiatric conditions from Alzheimer's disease[310], to traumatic brain injury[85,367], Pick's disease[368], synucleopathies[369], or late life depression[370]. The current data are the first supporting a beneficial effect of *APOE*-ε4 in a neuropsychiatric disorder.

The current findings support the hypothesis of an antagonistic pleiotropic phenomenon for the *APOE*-ε4 allele in FEP. As discussed, antagonistic pleiotropy is a concept coined by George Williams, an evolutionary biologist, that poses that some genes that affect fitness (i.e.

survival and reproduction) may latter have an detrimental effect during senescence[342]. The first condition for *APOE*-ε4 to have an antagonistic pleiotropic effect, increase of fitness early in life, is supported by research showing different *APOE* alleles having different fertility rates in humans[149,150]. In addition, there are data pointing to cognitive advantages early in life to support the argument of increased fitness associated with *APOE*-ε4. Wright and colleagues found that infant *APOE*-ε4 carriers had an advantage in early stages of development measured with the Bayley Scale of infant development compared to those who were non-*APOE*-ε4 carriers, suggesting an early-life brain development advantage of having *APOE*-ε4 allele[344]. Mondadori et al. genotyped *APOE* in more than 300 young healthy volunteers and tested them on a verbal memory test showing that not only did *APOE*-ε4 carriers significantly outperform non-*APOE*-ε4 carriers, but also that there was a "dose-response" effect as *APOE*-ε4 homozygotes, did better than *APOE*-ε4 heterozygotes[328]. They also demonstrated that brain activity during a verbal memory task was different in *APOE*-ε4 carriers in several brain regions including the hippocampus. Additional functional MRI studies by the group led by Filippini converged with those of Mondadory et al, who reported recruitment of additional frontal brain regions and greater hippocampal activation as the underlying mechanism of *APOE*-ε4 carriers performing better in an encoding verbal memory task[329,371]. In keeping with the concept that *APOE*-ε4 may have an antagonistic pleiotropic effect, more recent research in younger healthy volunteers using fMRI while performing cognitive tasks has demonstrated that cognitive performance declines with age in *APOE*-ε4 carriers[372], and in older healthy *APOE*-ε4 carriers the compensatory mechanism fails. The earlier advantages in neurocognitive performance recedes with age in the *APOE*-ε4 carriers providing evidence a change of the effect over time from beneficial (youth) to no benefit (middle age), and then detrimental effect (i.e. increased risk of AD in older adults).

The possibility of an antagonistic pleiotropic effect of *APOE*-ε4 in FEP is not only intriguing, but it could also have far reaching implications in our understanding of genetics in SCZ. Firstly, considering the fact that SCZ is a condition strongly associated with genetic risk(s) how could it be reconciled from an evolutionary perspective that genes that lead to such a devastating condition have been conserved and not selected out? Could it be that other genes than *APOE*-ε4 offer some subtle but relevant advantages during youth, but later on are associated with an accelerated senescence process? Although those questions are beyond the scope of the data presented in this chapter, they do generate a valid and plausible hypothesis that is testable in future studies.

The mechanism underlying the interaction between FEP and *APOE*-ε4 over time (i.e. verbal memory only improved in those patients who were *APOE*-ε4, not in CTRL either), merits further attention. These data do not support a practice effect to explain the improvement in verbal memory in FEP-*APOE*-ε4 carriers only. Several factors make the possibility of a practice effect to explain these results very unlikely. We used two alternate versions of the CVLT-II to minimize practice effects[373], and participants were randomly assigned to receive those versions in a counterbalanced way. Neither the healthy volunteers nor non-*APOE*-ε4 FEP patients had improvements on verbal memory over time. This study was conducted as a prospective observational cohort. Patients received clinical care naturalistically, and interventions were not systematic. However, all the FEP subjects received antipsychotic medication. There may have been a potential interaction between treatment and *APOE*-ε4 status leading to the improvement in verbal memory performance. There is potential for a weak-moderate effect of antipsychotics on neurocognitive impairment, which has been suggested in a number of studies[334,374–377], but has

not been consistently replicated[378,379]. The current data might offer a potential explanation that would reconcile otherwise divergent results.

What would be the mechanism underlying this interaction between antipsychotics and *APOE*-ε4 that would lead to improvement of neurocognitive impairment? As discussed in Chapters 1 and 2, the principal proposed mechanism by which *APOE*-ε4 results in deleterious effects to the brain in pathological states points to the decreased efficiency of *APOE*-ε4 to transport cholesterol and the impact that this has on cellular homeostasis and repair mechanisms in the nervous system[249]. A plausible link may be related to the effect that antipsychotic medications have on the stimulation of lipid metabolism in the brain. A research group lead by Steen conducted a series of experiments showing that several antipsychotics including clozapine, olanzapine, and haloperidol increased the expression of cholesterol metabolism genes such as APOE or ABCA1 in human brain neurons through the activation of the Sterol-Regulatory Element Binding Protein (SREBP)[161,162]. Interestingly, Dean and others treated a group of rats with haloperidol and found a decrease in levels of apoE, but increased levels of apoE in postmortem brain tissue of patients with SCZ treated with antipsychotics[127]. It is reiterated that the rodent apoE gene does not include the human *APOE*-ε4 allele as part of their genetic variation. Results reported by Dean and colleagues may be congruent with data by Steen and colleagues. Furthermore, the same group lead by Steen reported that human hepatic cells would also increase the expression of cholesterol metabolism molecules, leading them to hypothesize a connection between antipsychotic administration and metabolic side effects[380]. Interestingly, associations between changes in lipid profile associated with antipsychotic medications and clinical improvements have been previously observed[158,159]. This raises the question as to whether antipsychotic treatment might impact cholesterol transport efficiency as a function of

APOE allele and be associated with the improvement in verbal memory. We extensively explored associations between pharmacological interventions and performance on verbal memory, and found no association between type of medication or accumulated dose with verbal memory performance. In addition, our study did not include any data on lipid profiles. This hypothesis remains to be tested in future studies. Nonetheless, our findings suggest a complex relationship between antipsychotic treatment and cognitive improvement, and could offer a framework to interpret the literature about this matter and its controversies such as whether second generation antipsychotics are superior or not[334,381].

4.4.4 Verbal memory performance and *APOE*-ε4 effects on clinical outcomes

We explored whether the *APOE*-ε4 status was associated with outcomes on psychopathology, social functioning, and global clinical impression. *APOE*-ε4 status was not associated with any outcome measures during the study nor was there was any difference in terms of premorbid illness measures such as duration of untreated psychosis, age of onset, or age at first decline. Given the hypothesis of antagonistic pleiotropic, *APOE*-ε4 in this young population appears not be associated with a detrimental effect. Our investigation did not replicate the observation of less positive symptoms in *APOE*-ε4 carriers[82,119], but are convergent to findings from other groups[79,86,87]. In addition, our data provide further support of an association of cognitive performance and negative symptoms[382]. Further, the correlation between verbal memory performance at follow-up and functioning in the domain of social networking on the RFS in our study adds support and replicates existing literature[383].

4.4.5 Limitations

Our sample was composed only of young individuals, limiting our ability to test the hypothesis that *APOE*-ε4 carriers having variable cognitive performance at different age stages.

Longitudinal variance merits further research, and could be addressed in future studies by including a group of older adults afflicted with chronic psychosis. Our sample size was small in comparison to large consortiums investigating genetic effects in SCZ. The number of *APOE*-ε4 homozygotes did not allow us to test for a dose-effect of the *APOE*-ε4 allele and other more nuanced hypotheses. The current data are a complementary contribution to that of large consortiums in that our results could be a catalyst for a hypothesis of antagonistic pleiotropy to be tested in large consortium samples, rather than only using data-driven strategies.

Chapter 5: Conclusion

5.1 Dissertation synthesis

The intent of this thesis was to investigate the **pleiotropic**[p] effects of **apolipoprotein** varepsilon 4 on the SCZ **syndrome** using a **translational** strategy. The findings demonstrated that *APOE*-ε4 influenced several apparently unrelated phenotypes such as cholesterol metabolism, LDLR signal transducing pathway involved in synaptic plasticity, hippocampal volume, and verbal memory performance (Figure 5.1). In addition, the findings suggest that pleiotropic effects may be antagonistic. Specifically, while *APOE*-ε4 carriers show a decrease in hippocampal volume (i.e. a detrimental effect), FEP *APOE*-ε4 carriers showed improved memory over time (i.e. a positive effect). On the other hand, in our older sample of postmortem tissue the findings would indicate that *APOE*-ε4 might be associated with decreased expression of molecules involved in cholesterol metabolism and transduction of signal in the LDLR pathway associated with synaptic plasticity. Collectively, the findings would indicate that *APOE*-ε4 is an unspecific factor that may have some additive effects, but no interactions. The findings do not support *APOE*-ε4 as an etiological factor in SCZ disorder, but they do indicate that *APOE*-ε4 may modify the phenotypic expression of the disorder (e.g. hippocampal volume or verbal memory), and it may do so in complex and opposed ways (e.g. smaller hippocampi, but improvement in verbal memory over time). The experimental findings from the three parts lead

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[p] Pleiotropic: from Greek *pleion* "more than" and *trope* "to turn, to alter, to change".

Pleiotropic (gen): the production by a single gene of two or more apparently unrelated phenotypes

to the emerging question as to whether *APOE*-ε4 may be associated with a particular syndromic presentation in SCZ.

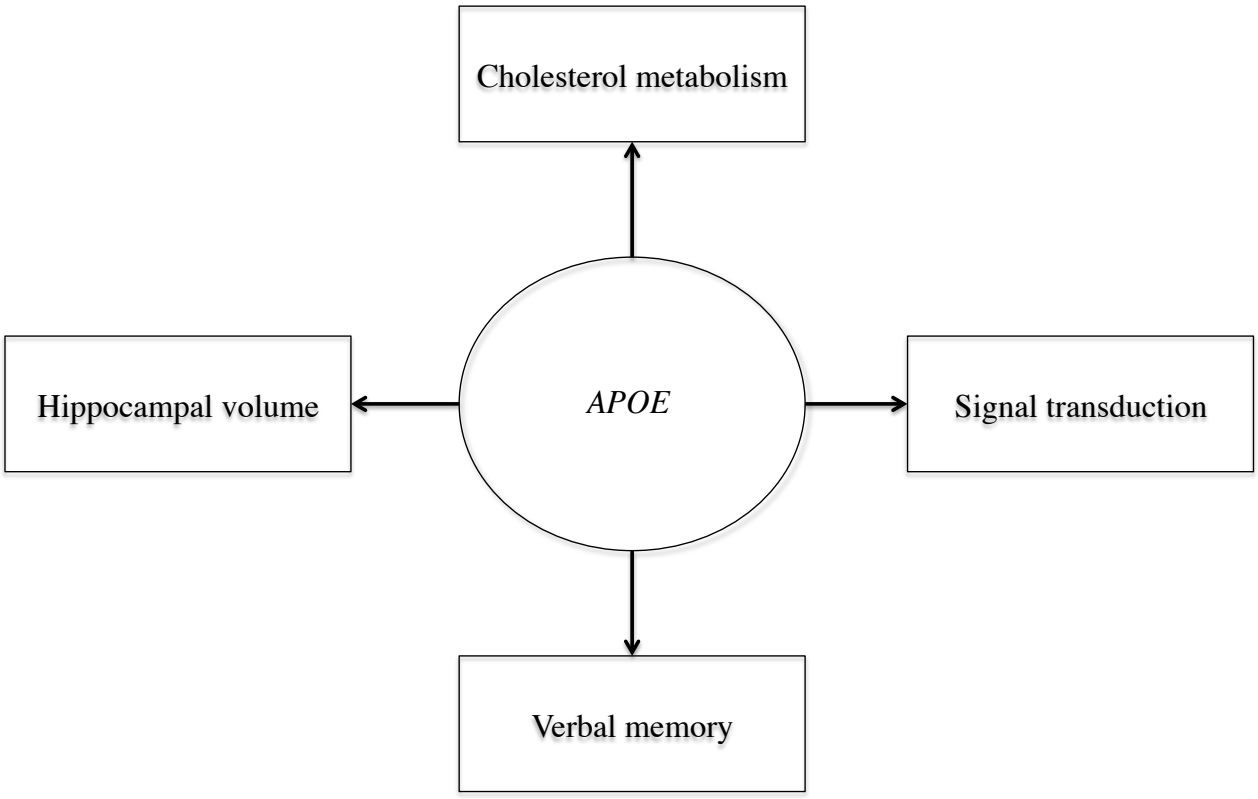

Figure 5.1 *APOE* influences multiple phenotypes: an example of pleiotropy

5.2 Main findings and conclusions

5.2.1 *APOE*-ε4, regulation of brain cholesterol metabolism, and apoER2/VLDLR signal transducing pathway

APOE genotyping and quantification of apoE protein, cholesterol, methylation of reelin promoter region, reelin, apoER2, and VLDLR was completed from human brain region BA9. The sample was from the Stanley Foundation repository and included 35 middle age, Caucasian patients with SCZ and 35 age-, sex-, ethnicity-matched CTRL. SCZ patients had a mean duration of illness of 20 years, had substantial lifetime exposure to antipsychotics and a quarter of them completed suicide as their cause of death. SCZ samples and control samples had similar postmortem intervals, storage time, and proportion of left/right hemisphere sampled. SCZ samples had lower brain pH than control samples.

Our findings indicated that there was no relationship between levels of any of the molecules quantified and clinical variables such as age of onset, duration of illness, or death by suicide. Similarly, the lifetime CPZ dose equivalent had no relationship to the levels of any of the molecules quantified. Our data indicated that increased brain pH was associated with lower levels of reelin methylation and higher levels of reelin mRNA. Increased storage time was associated with increased reelin mRNA, but with lower apoE levels. Given these findings potential confounding variables were controlled for during statistical analyses.

Our data demonstrated a higher level of methylation of the promoter region of reelin in SCZ, but no statistically different amounts of reelin in SCZ. Our findings replicate the increase in methylation of the reelin promoter region, but are not convergent with prior studies showing as well decreased reelin expression. Increased methylation of the promoter region of reelin is associated in animal studies to decreased expression of the protein as methylation is associated

with more compacted DNA strains and decrease access to initiate transcription. In addition, ApoER2 mRNA was decreased in SCZ sample. Our finding is convergent with Suzuki and colleagues[287] who found that apoER2 levels were reduced in SCZ patients treated with antipsychotics, and it also provides complementary evidence as the reduction of apoER2 in our sample is in the brain whereas Suzuki and colleagues was in peripheral lymphocytes.

The data also provide evidence about regulatory effects of *APOE* on apoE, methylation of reelin promoter region and reelin expression. Our findings indicate that *APOE*-ε2 is associated with higher levels of both apoE and reelin expression, as well as with lower methylation of the reelin promoter region. In addition, the data suggest that both *APOE*-ε3 and *APOE*-ε4 are associated with the reverse pattern of less apoE and less reelin expression, but more methylation of the promoter region compared to *APOE*-ε2. Findings regarding differences between *APOE*-ε4 and *APOE*-ε3 are not conclusive.

The data also provide evidence regarding neurophysiological aspects of cholesterol regulation. The data demonstrate that apoE is more abundant in grey matter than in white matter whereas the reverse pattern is true for free (esterified) cholesterol. In addition, the data demonstrate that higher levels of cholesterol are associated with lower levels of apoE, but this strong correlation is only present in healthy volunteers, indicating that neurophysiological regulatory mechanisms of cholesterol metabolism and transport may be affected in SCZ.

5.2.2 *APOE*-ε4 and FEP are associated with decreased hippocampal volume

A young, mostly Caucasian, sample of patients during their first episode of psychosis was recruited from Metro Vancouver area. Clinically, the most frequent diagnosis was SCZ spectrum

disorders, (77% of the sample), and they had a brief duration of illness and a mean duration of untreated psychosis of 2 years. With regards to medications, they were either antipsychotic naïve at the time of brain scan or with less than 8 weeks of exposure to antipsychotics, and their level of severity of was moderate/severe. A sample of healthy age-, sex-, and ethnicity-matched volunteers was also recruited and two brain scans obtained at a similar time interval as FEP.

The FEP and the control group were similar in age, sex, and ethnicity, but the control group had higher years of education as well as higher estimated premorbid IQ. When the FEP group was stratified into *APOE*-ε4 carriers and *APOE*-ε4 non-carriers, no differences were observed on age, sex, ethnicity, DUP, duration or severity of illness. Furthermore, both groups had a similar time exposure, dose, or type of antipsychotic medications. Our data indicated that there was no relationship between any of the variables listed above and hippocampal volume.

Our findings replicated that hippocampal volume is reduced in FEP, but furthered the field by also showing that *APOE*-ε4 carrier status is associated with decreased hippocampal volume, even though no interaction of gene by FEP was observed. Similarly, our results were convergent with prior literature reporting there is little progression of hippocampal volume reduction over time[384] as our data indicated that hippocampal volume did not change significantly over time in either group. However, visual inspection of marginal means in Figure 3.2 show that hippocampal volume of *APOE*-ε4 non-carriers in FEP would decrease over time, although this does not reach statistical significance, perhaps it is a finding worth following up on.

Our data did not show any relationship between clinical variables such as symptomatic severity or duration of untreated psychosis with hippocampal volume, arguing against a neurotoxic effect of untreated psychosis being expressed in hippocampal structural changes.

Similarly, our data showed that hippocampal volume did not predict verbal memory performance and there was no association of hippocampal volume to verbal memory performance.

Our findings confirm and replicate that FEP (and SCZ) are associated with brain structural abnormalities, namely reduced bilateral hippocampal volume, even during early stages of the disorder and that those are unlikely to be caused by the effect of antipsychotic medications. In addition, our data demonstrate that *APOE*-ε4 has also an impact on a different phenotypic expressions other than cholesterol metabolism (e.g. hippocampal volume), and that it does contribute to explaining a statistically significant variance of hippocampal volume, even though the effect size is not large. Our findings are intriguing because on one hand brain abnormalities are demonstrated, but on the other hand the impact of those brain abnormalities on clinical outcomes, including cognition, remains elusive. A plausible hypothesis to explain this apparent paradox is that volume as a proxy variable of the underlying etiopathogenesis may be too distal from the underlying molecular and cellular abnormalities that lead to volume reduction. Furthermore, *volume* as a biological variable is determined by multitude of factors at several epistemological levels (e.g. molecular, cellular, tissue). Therefore the ability of hippocampal volume to capture signal about how the brain works or the effect of a disease process such as schizophrenia may be limited.

Another aspect that remains unanswered and represents a gap of knowledge between post-mortem research and brain imaging is the origin of volume reduction. Post-mortem studies have demonstrated subtle abnormalities in the hippocampus, but the magnitude of those is not sufficient to account for that estimated ~4% volume reduction. If neurons or glia are not significantly reduced, the reduction in neuropil cannot account for it, and there is barely myelin in the hippocampus, what it the origin of volume reduction? Perhaps an unexplored area that

might yield some answers in this regard in neuroimaging is whether the water content or reduced blood flow may account for these volume reductions.

5.2.3 Verbal memory improvement in FEP *APOE*-ε4 carriers over time: an antagonistic pleiotropic effect?

A young, mostly Caucasian, sample of patients during their first episode of psychosis was recruited from the Metro Vancouver area. Clinically, the most frequent diagnosis was SCZ spectrum disorders, 67% of the sample, and they had a brief duration of illness and a mean duration of untreated psychosis of 2 years. With regards to medications, they were either antipsychotic-naïve at the time of cognitive testing or with less than 8 weeks of exposure to antipsychotics, and the level of severity of the sample was moderate/severe. A sample of healthy age-, sex-, and ethnicity-matched volunteers was also recruited and two neuropsychological assessments were conducted at similar time intervals as with FEP.

The FEP and the control group were similar in age, sex, and ethnicity, but the control group had more years of education as well as higher estimated premorbid IQ. When the FEP group was stratified into *APOE*-ε4 carriers and *APOE*-ε4 non-carriers, no differences were observed on age, sex, ethnicity, DUP, duration or severity of illness. Both groups had a similar time exposure, dose, or type of antipsychotic medications. The data indicated that there was no relationship between any of the variables listed above and verbal memory performance.

Our findings converge with existing literature showing verbal memory is significantly impaired in FEP patients, but differ in that neurocognitive impairment was similarly affected across all the verbal memory subdomains without a specific subdomain being particularly impaired. These results would suggest a more generalized impairment in verbal memory rather than a focalization on a particular subdomain or verbal memory process. It appears that verbal

memory deficits encompass impairments in acquisition of information, encoding, recall, and consolidation as well as both short-term and long-term memory systems (Figure 4.9).

At baseline, *APOE*-ε4 had no effect on verbal memory performance in FEP patients or CTRL although in this group visual inspection of the profiles in Figure 4.5 shows a separation of the profiles where *APOE*-ε4 carriers performed better; this was not statistically significant, but it important to recall that the group of 39 CTRL only had 8 who were *APOE*-ε4 carriers. In addition, *APOE*-ε4 status did not have any particular impact on any of the sub-domains within verbal memory.

Precisely due to the generalized deficit, the CVLT 1-5 total recall was used as the measure within CVLT that best condenses verbal memory globally to test the effect of *APOE*-ε4 over time. Our data showed an effect of diagnosis with FEP patients performing worse that CTRL. In addition, there was an interaction of diagnosis by *APOE*-ε4 status by time, where in FEP *APOE*-ε4 carriers verbal memory improved over time. This is a novel finding not reported before in the literature and would appear to be counterintuitive considering that *APOE*-ε4 is the main genetic risk factor for Alzheimer's disease as well as the results discussed regarding smaller hippocampal volume in *APOE*-ε4 carriers.

How is it possible that the same gene has an effect on two different phenotypes (i.e. hippocampal volume, verbal memory) of opposite direction (i.e. decreased hippocampal volume, but improved cognition)? The hypothesis is that *APOE*-ε4 may be associated with an *antagonistic pleiotropic* effect. This phenomenon postulates that a gene may be associated with different phenotypes, and these effects may be antagonistic. From an evolutionary perspective, the antagonism translates to a positive effect in the form of increased fitness, and the negative effect to a decreased chance of survival. Our findings would be convergent with data showing an

increase rate of fertility associated with *APOE*-ε4 and some neurocognitive advantage in youth carrying the *APOE*-ε4 allele. Our findings are the first to report this effect in a disease state, and have significant potential implications on our understanding of the etiopathogenesis of SCZ. Given the increase in human lifespan in the last 200 years to currently ~80 years old in developed countries[385], it has been challenging to reconcile that genes associated with such a severe condition have been maintained through natural selection. Human lifespan had been significantly shorter for millennia, when presumably natural selection occurred, leading to the contemporary genetic pool[385]. Human lifespan had been estimated to be ~25-30 years prior to the 17th century and therefore natural selection would have driven the selection of genes that increased survival and fitness in that short lifespan[385]. The recent advent of increasing senescence has uncovered that some of those genes selected associated with an advantage early on are detrimental during senescence. This framework would indeed be a very plausible model to understand how genes that are so detrimental during senescence have been selected during natural selection. This conceptual framework has received support in the literature from research groups arguing that SCZ may be conceptualized as a disorder of accelerated senescence[386].

5.3 Limitations and strengths

This thesis provides important translational insights into the etiopathogenesis of SCZ using the *APOE*-ε4 allele as a test model. This approach comes with caveats and limitations. First, even though the sample sizes are large in absolute numbers for postmortem studies as well as first episode psychosis cohort studies, the *APOE*-ε4 allele frequency was only ~25%, as expected. The statistical power to detect small differences is limited and the possibility of type II errors should be considered. Conversely, positive findings associating *APOE*-ε4 to effects on protein levels, hippocampal volume reduction, and cognition would represent strong evidence of

APOE-ε4 effect. These findings represent an important first step using this approach for future research to qualify, refute, or replicate and confirm the findings. An additional encompassing caveat of this thesis is the scope associated with a translational approach. This thesis deals with a wide array of knowledge that goes from genetics, proteomics, and metabolomics in chapter 2 (basic science level), and then transitions to more complex levels such at the level of tissue or system in chapter 3 with neuroimaging work, and finally to a further complexity level with behavior in chapter 4 addressing neurocognitive deficits. This broad scope necessarily leaves gaps in knowledge within each level of complexity, as well as in the transitions to more complex and emergent levels of knowledge. In spite of these gaps, exploring new approaches and testing new boundaries are inherent challenges that are worth undertaking, and the data do provide logical connections between the epistemological levels and represent a valuable first step in conducting translational research.

A caveat specific to chapter 2 is that postmortem studies capture phenomena in a static fashion, and also are the last snapshot of a sequential chain of events. This poses a limitation with regards to the control of potential variables of interest pre-mortem (e.g. the presence of lipid-lowering medications in our sample), and it also does not allow for the study of dynamic phenomena the same manner that *in vitro* or animal studies allow. Human postmortem studies are essential when placed in context with other approaches, as they allow for testing specific questions in the relevant system and may be more directly translated. An example is the finding that apoE levels were not reduced in SCZ patients who were exposed to a significant lifetime dose of antipsychotics. Prior research reported a significant reduction in apoE levels in grey matter in rats with rodent APOE gene and rodent apoE isoform treated with haloperidol[127], but the data did not converge with those in rodents. Rodents do not have the three common human

allele variants for *APOE* and this difference has an impact on apoE and cholesterol metabolism in rats. The current results have the potential to inform future research to use genetically modified animal models with humanized *APOE* alleles to test the effect of antipsychotics.

A specific caveat to chapter 3 is related to the use of total volume as the only morphometric dependent variable of study. Volume is one of many morphometric properties of brain structures and mainly captures the dimension of size; it tells us how large or small a structure is. In *In vivo* MRI, the sizest of brain structures measured are likely to be determined by the amount of cellular bodies (neurons, glia, endothelial cells, cells circulating in blood vessel), their cytoplasmic extensions (i.e. axons, dendrites, etc.), the amount of water, blood, and a mixture of molecules in the neuropil composed of lipids, proteins, etc. Volume as a variable *per se* may not inform what is the specific origin of volume increase or decrease. As such, hippocampal volume reduction in *APOE*-ε4 carriers does not illuminate the precise mechanisms that result in *APOE*-ε4 carriers having decreased volume. The study of complementary morphological features such as shape or hippocampal subregions may offer additional information to address this question. Similarly, hippocampal volume may be too distal from the underlying mechanisms of hippocampal function that are related to behavioral outcomes such as verbal memory. Volume reductions in the cornu ammonis (CA1) and dentate gyrus were reported in one study[223], although this was not replicated in another study that reported CA2/3 and CA4/DG deficits[319]. Divergent findings might be explained by more recent data showing that hippocampal abnormalities in SCZ might start in CA1 and expand to other areas in the hippocampus as the illness progresses[225].

Only using a sample of FEP is also associated with limitations, as comparing variables of interest between young and older samples is not possible. This might have been particularly

useful to directly test an antagonistic pleiotropic effect in a single sample (i.e. verbal memory in older adult *APOE*-ε4 carriers). The data enable future studies to test this hypothesis. Last, our sample size was associated with a small number of APOE-ε4 homozygotes, and therefore it did not allow us to test for a dose-effect of the APOE-ε4 allele. The current data are a complementary contribution to that of large consortiums, as our results may generate specific hypotheses regarding pleiotropic effects that may be tested in large consortium samples.

5.4 A translational perspective of findings: from molecules to brain structure to neurocognitive performance

A complex system consists of multiple interconnected and interwoven parts, and to understand it, it is not only essential to know its parts, but it is also critical to understand how those parts are interconnected and influence each other[387]. Complex systems have certain properties in common such as i) contain many parts interacting nonlinearly; ii) parts are interdependent; iii) the system encompasses several scales, and iv) is capable of emergent behavior; and emergence occurs when a phenomenon on a scale cannot be explained simply by the addition of the properties of its parts[388]. In addition, biological systems are a particular type of complex system: they are adaptive systems as they are capable of self-organization and self-reproduction. In Figure 5.2 a representation of complex systems and emergence is represented.

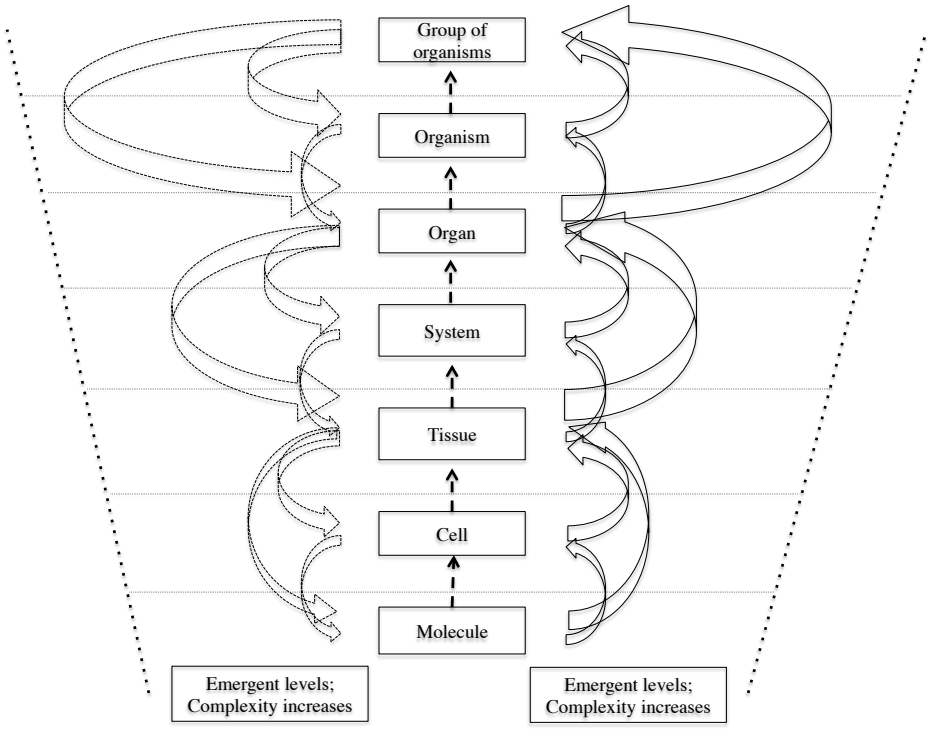

Figure 5.2 Complex systems and emergence

In the context of complex systems, a translational medicine approach may be considered as a strategy to address questions that encompass several levels in a complex system, and aims to shed light on the interdependencies and interconnections between levels.

The encompassing aim of this thesis was to conduct a translation medicine approach, and the key contribution is to demonstrate that this approach may lead to critical insights about the etiopathogenesis of complex disorders such as SCZ (Figure 5.3). One of the specific applications of this thesis is the implementation of this model to the study of other etiopathogenic mechanisms in SCZ, to shift the focus from the idea of single cause and to embrace complexity as a valid construct. The identification of specific and complex etiopathogenic mechanism(s) may lead to more specific nosological boundaries and, eventually, to true disease diagnoses. This

paradigm shift may have profound implications as well to therapeutic interventions whereby treatments can be designed and aim at specific etiopathogenic mechanisms.

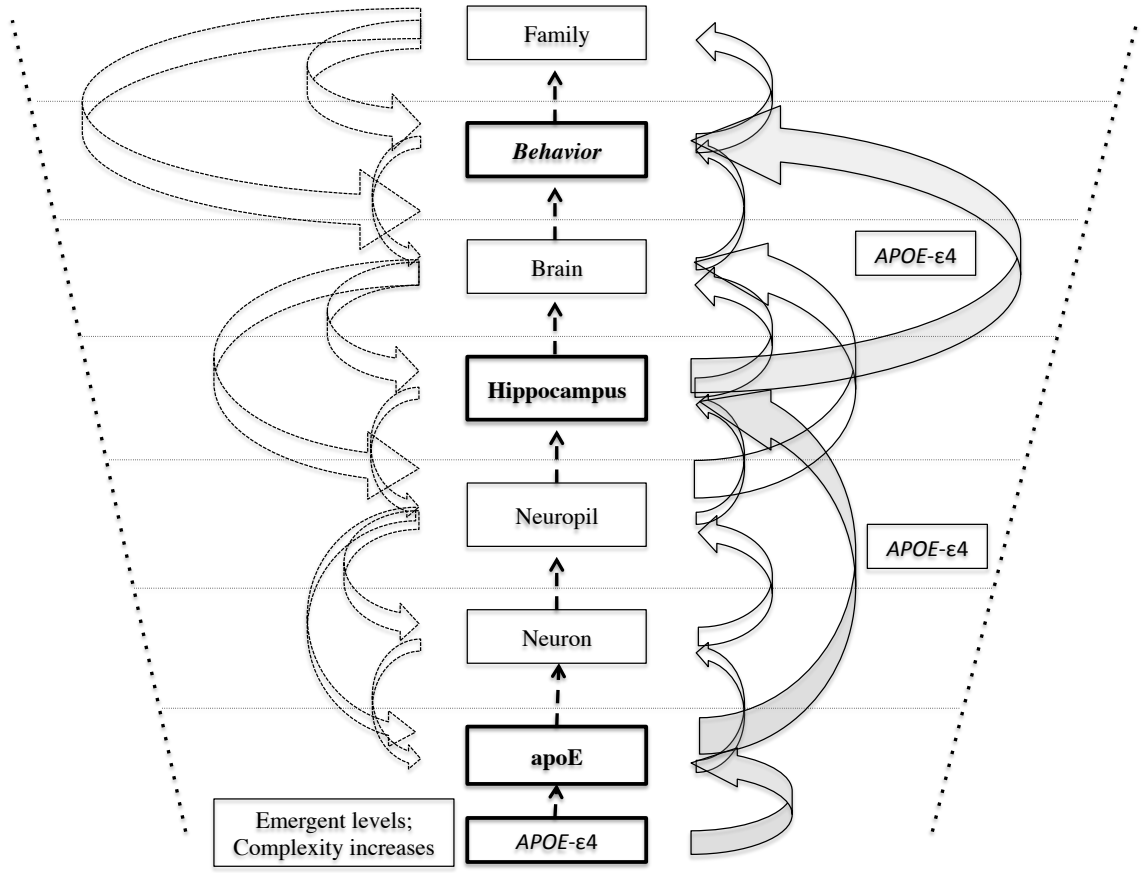

Figure 5.3 Investigating the pleiotropic effects of the apolipoprotein varepsilon 4 allele on the SCZ syndrome: a translational medicine thesis

The thesis addressed questions at the **molecule level**: using human post-mortem sample of BA9 from patients with chronic SCZ and CTRL was used to address questions related to protein expression of apoE and related molecules in the LDLR signaling pathway, as well as genotypic regulation of *APOE* on these molecules. Subsequently, the thesis addressed a **system level** (hippocampal volume), and the link to *APOE*-ε4 by analyzing a sample of first-episode psychosis and healthy volunteers genotyping *APOE* and measure hippocampal volume. Last, the **behavior level** was addressed measuring verbal memory and the influence of *APOE*-ε4

5.5 Future directions

The findings on memory improvement over time in FEP *APOE*-ε4 carriers raise the question of what was the factor that interacted with *APOE*-ε4 during that period that lead to the improvement. As discussed in chapter 4, a plausible hypothesis is an interaction with antipsychotic medications and their influence on cholesterol metabolism. Although this hypothesis needs to be tested to draw firm conclusions, it would be plausible to think that changes in cholesterol metabolism in the brain might have more potential to address cognitive symptoms than changes in dopamine levels. Perhaps there are potential pharmacodynamics mechanisms beyond the neurotransmitter-receptor profile strategy that might prove to address some symptom domains in SCZ. Novel treatments such as repetitive transcranial magnetic stimulation have shown improvements in cognition[389,390], and this effect may be related to its effect on synaptic plasticity, rather than a neurotransmitter-receptor mechanism. A feasible way to conclusively test the antagonistic pleiotropic hypothesis of *APOE*-ε4 effect on cognition and hippocampal volume in schizophrenia would be to leverage ongoing large consortium aggregating imaging and genetic data from multiple studies and sites such as the ENIGMA consortium[391].

Cholesterol metabolism in the brain is essential for a healthy developmental trajectory and homeostatic maintenance. Therefore, cholesterol metabolism and homeostasis is a plausible factor at play in the context of the developmental hypothesis of schizophrenia. Epidemiological data showing age of onset of psychosis coincides with late stages of myelination in late adolescence may be a clue to such connection[236,392–394], and future research may further explore

the role of cholesterol metabolism and cholesterol homeostasis and the emergence of psychotic symptoms in schizophrenia[395].

Future experiments may also follow up on recent data demonstrating that apoE4 expressed in GABAergic interneurons is associated to learning and memory deficits in rodents, and that conditional deletion of apoE4 in GABAergic interneurons provides a protective factor to such detrimental effect. Of note, the deleterious effect is not observed when apoE4 is of astrocyte origin[396]. This finding may be particularly relevant to schizophrenia as GABAergic interneuron abonormalities have been posited to play a critical role its etiopathogenesis[397].

This thesis is ambitious in scope as it encompasses experimental work at two stages across the lifespan (youth and middle age), as well as different levels of complexity (i.e. molecules, brain structure, and behavior). This approach illuminated some questions, but it also showed gaps in knowledge that prevented making conclusive claims along the two dimensions of complexity and time. It represents the first step to a new approach in research. Future designs aiming at addressing these two dimensions might require of long-term follow-up studies where measures at different levels of complexity are taken repeatedly. Clinical research in SCZ over the last 60 years has mostly conducted cross-sectional studies or short-term follow-up studies, and the few exceptions with longer follow up intervals have not combined measures at different levels of complexity. There is a gap and a need to undertake these types of studies to address how phenomena may change or evolve across the lifespan (Figure 5.4.), and to include measures at different levels of complexity (molecules, brain structure, behavior, etc.).

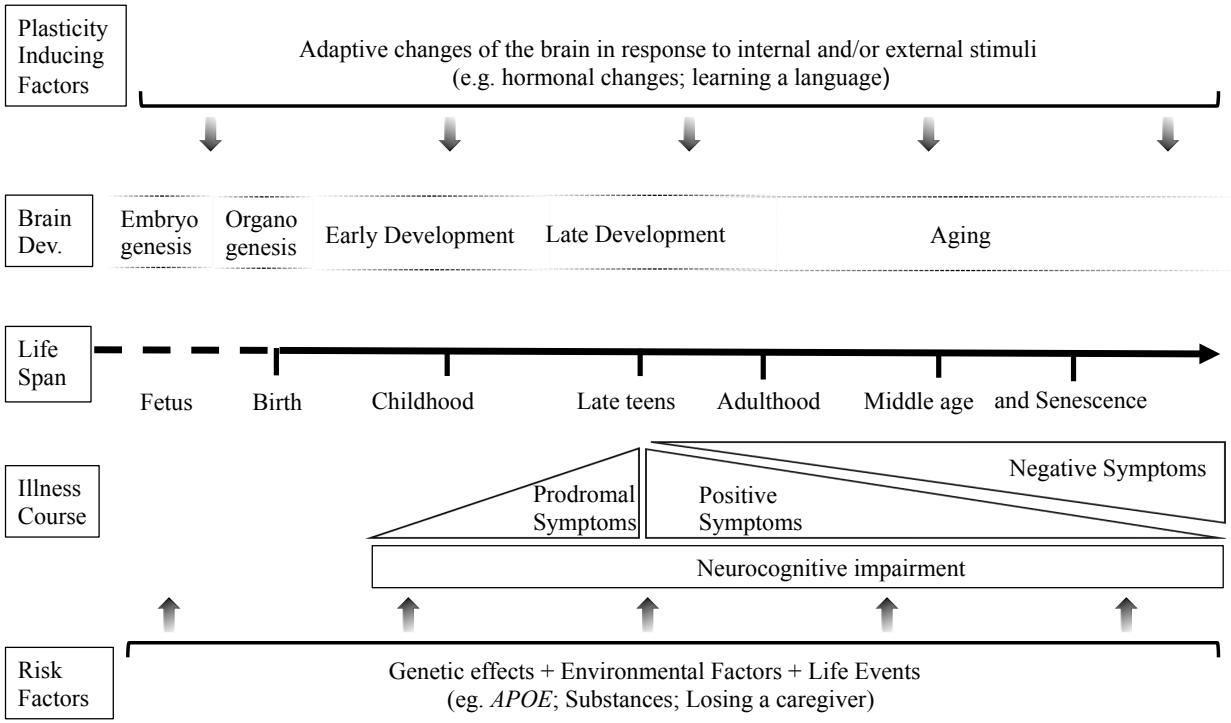

Figure 5.4 A diachronic model of schizophrenia: a roadmap for future research

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Appendices

Appendix A Detailed cognitive testing protocol

A.1 North-American reading test (NAART)

Administration:

The following script is read to the subject:

"*I want you to read slowly down this list of wards starting here (indicate debt) and continuing down this column and on to the next. When you have finished reading the words on the page, turn the page over and begin here (indicate top of second page). After each word please wait until I say "next" before reading the next word. I must warn you that there are many words that you probably won't recognize, in fact most people don't known then, so just guess at these ok? Go ahead*."[320]

There is no time limit to complete the task and the subject should be encouraged to guess, and all responses should be reinforced. The subject may change a response if they wish, must make it clear which version is their final choice.

A.2 Kauffman brief intelligence test[321] (K-BIT)

Administration:

The K-BIT has three subtests: two to estimate verbal IQ and one for non-verbal IQ: The vocabulary subtest is composed of 60 items each of them consisting of an array of six full-color illustrations or pictures. The tester says a word or asks a general- information question, and the subject points to the picture that shows the meaning of the word or the answer to the question. The riddles subtest has 48 items that aim at measuring verbal reasoning, and vocabulary knowledge. The tester asks a riddle, and the subject either point to a picture that shows the answer to the riddle or says a single word that answers the riddle.

A.3 California verbal learning test[320] (CVLT)

Administration:

In this test, 16 words (list A), drawn from four different semantic categories, are presented five times (Figure 4.1). Both word lists are introduced as shopping lists as this is an ordinary task people often encounter and thus may better inform learning strategies[398]. Immediately after the fifth trial, a new list (list B) is read to the subject and they are asked to repeat as many words as possible. A short delayed test (CVLT-SD) is presented immediately after recall of list B, where the participant is asked to recall the words of list A, first without cues ("free"), and then with cues. A long delayed recall test (CVLT-LD) is presented after an interval of ~20 min where the subject may continue with other non-verbal tasks. Similar to the short-delay test, the long delay test is presented first without cues ("free"), and then with cues. Finally, a "yes–no" recognition test that consists of the 16 items from list A, 16 from list B and 16 random distractor items is presented. In the present study, we included the total number of words recalled across the five learning trials ("learning"), the number of words recalled immediately after list B ("short delayed free recall") and after the long delay ("long delayed free recall"), and the number of hits on the recognition trial ("recognition"). Of note, the instructions do not warn subjects of the delayed recall and recognition tasks. While giving instructions or not giving them did not make a significant difference in college students, when giving subjects a longer word list providing a warning about delayed tasks did make a significant difference on delayed recall performance[399]. It would then be plausible to expect that advance warning would increase the delayed recall and recognition scores in clinical settings where there is less ceiling effect.