Genomic Press

Genomic Psychiatry

Advancing science from genes to society

Volume 1 | Number 1 | January 2025

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SCOPE: *Genomic Psychiatry* has a broad scope. As our goal is to interweave genetics with other advances in contemporary psychiatry, we welcome innovative research from in-depth studies of psychiatric genomics to broader investigations of the underpinnings, treatments, outcomes, and consequences of mental health. In addition to the genetic aspects of mental illness, our scope includes advances in neuroscience of potential relevance to mental illness, imaging, psychology, pharmacology, therapeutics, microbiology including the microbiome, immunology, endocrinology, brain stimulation, functional neurosurgery, "big data," computational approaches including artificial intelligence (AI), epidemiology, and public health initiatives.

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Cover Art

The cover highlights key elements of the fragile X syndrome aticle by Sakamoto et al. featured in this issue. DNA strands represent the FMR1 gene, which when silenced leads to fragile X syndrome, a condition associated with high rates of autism spectrum disorder. Laboratory mice, widely used as models for studying this condition, are shown alongside the computational structures of neonatal social communication (visualized as colorful dot clusters) and the structure of bumetanide central to understanding the disorder. This body of work demonstrates that prepartum bumetanide treatment can reverse altered neonatal social communication patterns in a mouse model of fragile X syndrome. The study provides important insights into how Fmr1 deletion affects distinct elements of early vocalization and later social interaction, suggesting potential developmental windows for therapeutic intervention. For further information on this topic please see the paper by Sakamoto et al on pages 61–72.

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Genomic Psychiatry

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EDITORIAL

Inaugural Editorial – Introducing *Genomic Psychiatry*: Advancing science from genes to society

Genomic Psychiatry January 2025;1(1):1–2; doi: https://doi.org/10.61373/gp024d.0004

We are delighted to introduce *Genomic Psychiatry*, a new and groundbreaking medical research journal that aims to revolutionize the field of mental health. Unlike traditional genetics journals, *Genomic Psychiatry* will bridge the gap between genes and the vast array of interconnected disciplines that contribute to our understanding of mental health, advancing science from genes to society.

In recent years, the field of genomics has made significant strides in unraveling the genetic basis of psychiatric disorders. Yet, our editorial conviction is that far more monumental advances will emerge from a nuanced examination of the unbroken spectrum extending from genetics to 'omics sciences, neuroscience, cognitive behaviors, medical imaging, clinical psychiatry, pharmacotherapy, controlled clinical trials, and the far-reaching societal implications of mental well-being (1) gene-enviorment interactions, social and environmental exposures.

Recognizing the multifaceted nature of mental health, *Genomic Psychiatry* will publish articles that not only delve into genetics and genomics but also embrace a wide range of related topics. We encourage submissions that explore the interplay between genetic markers, environmental factors, social determinants, and resultant mental health profiles (2, 3). From cutting-edge research on the role of epigenetics in psychiatric disorders to studies investigating the social determinants of mental health, we welcome a diversity of perspectives and methodologies.

By embracing a comprehensive approach, *Genomic Psychiatry* will provide a platform for researchers to showcase their work at the intersection of various disciplines (4). This journal will foster collaboration and inspire novel insights, thereby propelling the field forward. We firmly believe that the future of mental health research lies in embracing a multidimensional approach that integrates genetics, genomics, and beyond.

To ensure inclusivity and foster innovation, *Genomic Psychiatry* invites submissions from researchers and clinicians working across the entire spectrum of mental health. We encourage authors to explore the application of genomics and genetics in clinical psychiatry, pharmacological interventions, and treatment trials (5). We are also interested in digital medicine, e-health, and the use of artificial intelligence in computational psychiatry (3). Additionally, we welcome papers that investigate the neurobiological underpinnings of psychiatric disorders, advancements in brain imaging techniques, and behavioral studies that shed light on the complexities of mental health.

As the field of mental health continues to evolve, it is imperative that we create a space that encourages translational science, interdisciplinary collaboration, and knowledge exchange (6). *Genomic Psychiatry* endeavors to be that space, where scientists, clinicians, and researchers from various backgrounds join forces to explore the intricate nature of mental health disorders.

Our editorial board, comprising so far 50 esteemed members, stands as a cornerstone of *Genomic Psychiatry's* strength. These members are not just eminent in their fields but also globally recognized for their contributions. Among them is Nobel Laureate Thomas Südhof, underscoring the board's prestige. Several members distinguish themselves with affiliations to esteemed institutions: in the US National Academy of Sciences, we have the likes of Huda Akil, Moses Chao, Fred Gage, Jan-Ake Gustafsson, Yasmin Hurd, Robert Malenka, John Rubenstein, and again Thomas Südhof. Within the US National Academy of Medicine, our roster includes Fred Gage, Raquel Gur, Yasmin Hurd, Kenneth Kendler, Robert Malenka, Maria Oquendo, John Rubenstein, Alan Schatzberg, Thomas Südhof, Gustavo Turecki, and Myrna Weissman. The Royal Society honors Jonathan Flint and John Hardy as its Fellows. Additionally, the British monarchy has knighted two of our board members, John Hardy and Michael Owen, in recognition of their extraordinary achievements. This diverse and accomplished group mirrors the unparalleled expertise and global recognition our editorial board enjoys.

We would like to highlight our **Innovators and Ideas** section that spotlights individuals who have made noteworthy contributions to the field. Four of our editorial board members have already contributed to this exciting section as research leaders: Maria Oquendo (neurobiology and clinical approaches to suicidality) (7), Gustavo Turecki (trauma, depression, neuropathology, and genomics) (8), Anthony Grace (brain circuits, schizophrenia, and depression) (9), and Noboru Hiroi (neurobiology of human copy-number variation) (10).

We invite you to embark on this exciting journey with us. Together, let us unravel the mysteries of the human mind, leveraging the power of genomics, genetics, and the wealth of scientific disciplines that converge upon the realm of mental health. Join us in redefining the landscape of psychiatric research and fostering a better understanding of mental health for the benefit of individuals and society as a whole.

Welcome to *Genomic Psychiatry* – where the genetic, the behavioral, the environmental, and the societal merge to develop new paths towards optimal mental health.

Julio Licinio[1]

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GUEST EDITORIAL

Real-world implications of the prospects for prevention of clinical Alzheimer's dementia

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On Valentine's Day 2024, *The Los Angeles Times* published a story entitled, "Inside the plan to diagnose Alzheimer's in people with no memory problems—and who stands to benefit" (1). The story focuses on the financial implications for drug companies and patient advocates. The National Institute on Aging's AHEAD345[1] study (2) is a legitimate, federally funded, randomized clinical trial that is designed to the highest clinical research standards. This is a proven method for new drugs to be put to the most rigorous test using a placebo-controlled, double-blind, state-of-the-art design. The AHEAD345 trial will test the possibility that clinical Alzheimer's dementia may be preventable if diagnosis and intervention are triggered by blood-based biomarker changes detected at midlife despite an absence of symptoms. There is now clear evidence that Alzheimer's pathology develops 20 or more years prior to the appearance of clinical symptoms. The *Times* piece emphasizes that the effort to seize upon this potential window for intervention is at least partially motivated by the prospect that drug companies and dementia advocacy groups will be financially enriched if trials like AHEAD345 succeed. The *Times* article avoids words like "breakthrough" and "moonshot" that are frequently used to describe the mitigation or elimination of major illnesses such as cancer, diabetes, heart disease, and AIDS.

The cost of bringing each new prescription drug to market is estimated at $350 million (3). Usually, generating profits to underwrite ongoing research has been accepted as a sound business model and viewed as evidence of the entrepreneurial spirit of scientists and clinicians. Why was the development of blockbuster drugs that prevent clinical manifestations of atherosclerosis welcomed, while the prospect of preventing dementia is viewed in the first analysis as primarily profit-driven? Is the suffering of younger persons with atherosclerosis and cancer more important than the suffering of elder persons living with dementia and their families? Are scientific discovery and financial profitability mutually exclusive? I would have predicted that any tension between these two outcomes would be a small price to pay if we eliminate an illness that costs 2 trillion dollars per year in the US alone (4).

Many breakthroughs enrich inventors. The Nobel Prize has a monetary value of 11 million Swedish kronor (5). Rigorous trials of drugs are essential, regardless of who stands to benefit either financially or emotionally. While it is entirely reasonable that skeptics hold inventors' feet to the fire, the inventors should be entirely open to scrutiny to realize the common goal of authentic, valid, reproducible data. Skepticism about the outcome does not mean that audacious and potentially lucrative hypotheses should not be tested. The unpredictability of science is the essence of why experiments are conducted.

One goal of the RAND Corporation is to elucidate how successful dementia treatment and prevention might modify the clinical and economic landscape in a range of situations (6). RAND recognizes the substantial variation in the capacities of various healthcare systems to detect, diagnose, and treat or prevent early-stage Alzheimer's with disease-modifying treatments (DMTs). The estimated wait times and the number of patients treated are sensitive to the uptake of brief cognitive assessments by the public and by primary care providers. The estimated average wait times vary by state and can be three times longer in rural areas than in urban areas. Care models that enable primary care physicians to diagnose and evaluate patients for treatment eligibility would significantly reduce wait times for specialists and increase the number of people treated from 2025 through 2044. Improved triage of patients using blood-based biomarker tests could further reduce caseloads for specialists. Widespread delivery of Alzheimer's DMTs will require a combination of strategies to (1) communicate the value of detection and treatment to patients, (2) integrate primary care physicians into the detection and diagnosis pathway, and (3) address capacity disparities across the United States and around the world. These challenges for implementation can only be afforded if DMTs generate enough resources to offset this increase in demand. Critics with legitimate concerns should allow for the possibility that the potential profitability of breakthroughs does not mean that we should avoid asking whether prevention is possible.

Thorny questions remain to be answered. Trials have not been adequately inclusive and diverse (7). Standards for minimum clinically significant benefit are still under development, both for persons living with dementia and for their caregivers (8). Nevertheless, there is no reason not to begin sorting through these implications so that we are appropriately prepared if Alzheimer's prevention succeeds. Current evidence suggests that as many as 76% of patients receiving subcutaneous lecanemab (vs 55% of patients receiving placebo) have a complete arrest of their cognitive decline (9). On what planet is this a bad outcome?

Sam Gandy, MD, PhD[1,2]

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Competing Interests

Dr. Gandy is a co-founder of Recuerdo Pharmaceuticals. He has served as a consultant in the past for J&J, Diagenic, and Pfizer, and he currently consults for Cognito Therapeutics, GLG Group, SVB Securities, Guidepoint, Third Bridge, MEDACORP, Altpep, Vigil Neurosciences, and Eisai. He has received research support in the past from Warner-Lambert, Pfizer, Baxter, and Avid. He currently receives research support from the NIH and the Cure Alzheimer's Fund.

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[1]Acronym for an NIH clinical trial of blood-based biomarker guided treatment with anti-amyloid antibody or placebo.

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INNOVATORS & IDEAS: RISING STAR

Natalia Acosta-Baena: The genetic gap between neurodevelopment and neurodegeneration

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Keywords: SPAG9, JIP4, retrograde axonal transport, polygenic, pleiotropy, Intellectual disability, dementia, genetics, epidemiologist, neurodevelopment

Dr. Natalia Acosta-Baena embodies the rare confluence of clinical and basic science expertise that modern neuroscience demands. As a physician-scientist at the University of Antioquia's Neurosciences Group, she combines her medical training with a master's in clinical epidemiology and doctoral studies in basic biomedical sciences, specializing in Genetics. Her groundbreaking work began with contributing to characterize the world's largest population affected by autosomal dominant genetic Alzheimer's disease (mutation E280A in PSEN1), establishing a foundation for numerous studies on biomarkers, clinical trial design, and genetic modifiers in this pivotal cohort. In a landmark discovery, Dr. Acosta-Baena's research revealed a novel syndrome linked to a SPAG9 variant, demonstrating how a single gene involved in neuronal retrograde transport can drive neurodevelopmental problems and neurodegeneration in affected patients. This finding challenges the traditional separation between these processes and suggests shared biological pathways. Through her continued work with families affected by neurodevelopmental disorders, she has uncovered genetic networks that reshape our understanding of rare brain diseases. Her current translational medicine and genetic epidemiology research focuses on further exploring these unexpected connections between neurodevelopment and neurodegeneration. In a Genomic Press Interview, Dr. Acosta-Baena shared her life beyond the laboratory – from her early fascination with the human brain to finding joy in Colombia's mountain sunsets with her husband and son and drawing inspiration from Latin American writers like Cortázar and García Márquez. Her dedication to scientific rigor and human connection is reflected in her philosophy that each failure teaches something essential as she works toward translating genetic discoveries into meaningful healthcare policies and personalized medicine approaches.

Figure 1. Natalia Acosta-Baena, MD, MsC, PhD(c), Universidad de Antioquia, Colombia.

Part 1: Natalia Acosta-Baena – Life and Career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

It may sound strange, but ever since I can remember, I have wanted to study medicine and understand our brain. I do not have parents who were doctors or anyone in my family to influence me. It has motivated my life since I was little. I remember that I didn't like going to school, but I understood that it was the only way to get to work in what I was passionate about.

We would like to know more about your career trajectory leading up to your current role. What defining moments channeled you toward this opportunity?

A motivation: neuroscience research. Two opportunities: I started by attending a study group in Neuroanatomy with the Neuroscience Group of the University of Antioquia, and I accepted the opportunity to do a master's degree in epidemiology with the same research group with Professor Francisco Lopera and then complete a doctorate in genetics.

Please share with us what initially piqued your interest in your favorite research or professional focus area.

The complexity of human thought and action and the search for the evolutionary question of what the brain of homo sapiens had genetically and physiologically made us the species that managed to survive above the other hominids.

What impact do you hope to achieve in your field by focusing on specific research topics?

The greatest impact I hope to achieve with my research is to foster a collaborative effort that reaches individuals who are sick and their caregivers. I envision a future where our knowledge empowers people and

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influences health policies towards true prevention. Our studies, currently in the form of articles, hold the potential to be applied to the general population, shaping decisions and responses for patients.

Please tell us more about your current scholarly focal points within your chosen field of science?
Neurodevelopmental genetics is a field where we only see the tip of the iceberg. We do not see what we do not understand. When we manage to understand the relationship between genetic networks, we will be able to decipher neurodevelopment and neurodegeneration. My focus is genetics for prevention and personalized and community medicine.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences that you uphold within your research environment?
Continue with the medical consultation. Each rare clinical presentation of a disease that surprises is what motivates new questions.

At Genomic Press, we prioritize fostering research endeavors based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
I am passionate about new questions and new challenges. I am bored by absolute certainty and those who believe they have it.

What do you most enjoy in your capacity as an academic or research rising star?
That I do not feel like one.

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
I always enjoy the view of the sunset or the rain in the mountains of Colombia with my husband and son.

Part 2: Natalia Acosta-Baena – Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
There is not one, both words are a pleonasm.

What is your greatest fear?
"Without music, life would be a mistake" "Ohne Musik wäre das Leben ein Irrtum" (Friedrich Nietzsche, *Die Götzen-Dämmerung – Twilight of the Idols*, section: "Sprüche und Pfeile" (Maxims and Arrows) aphorism #33, 1895).

Figure 2. Natalia Acosta-Baena and her son embracing the beauty of the Antioquia Mountains during Colombia's COVID-19 lockdown (25 March 2020). Against a backdrop of lush tropical vegetation and bamboo groves characteristic of the region's mountainside, they stand on a cleared hillside slope, arms outstretched in a moment of joy and freedom despite the global pandemic restrictions. The contrast between the cultivated slope in the foreground and the dense forest canopy above captures the typical landscape mosaic of the Andean countryside.

Which living person do you most admire?
To every woman in Latin America who prioritizes her time for the integral education of her children above any other need.

What is your greatest extravagance?
Be happy.

What are you most proud of?
Being a mother.

What is your greatest regret?
I do not regret anything so far.

What is the quality you most admire in people?
Honesty and good humor.

What is the trait you most dislike in people?
Arrogance and prepotency.

What do you consider the most overrated virtue?
None. Each virtue is relevant to humanity, and research on humans and animals is an example.

[1] In the late nineteenth century, various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. In 2003 Proust's handwritten answers were auctioned off for $130,000. Multiple other historical and contemporary figures have answered the Proust Questionnaire, including among others Karl Marx, Oscar Wilde, Arthur Conan Doyle, Fernando Pessoa, Stéphane Mallarmé, Paul Cézanne, Vladimir Nabokov, Kazuo Ishiguro, Catherine Deneuve, Sophia Loren, Gina Lollobrigida, Gloria Steinem, Pelé, Valentino, Yoko Ono, Elton John, Martin Scorsese, Pedro Almodóvar, Richard Branson, Jimmy Carter, David Chang, Spike Lee, Hugh Jackman, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions, an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

What is your favorite occupation (or activity)?
Writing – and I can – dancing.

Where would you most like to live?
In Colombia, in my current house in a rural area in the mountains.

What is your most treasured possession?
My memories.

When and where were you happiest? And why were so happy then?
When my son was born, 10 years ago, and all the good times since then.

What is your current state of mind?
I currently feel calm and grateful for everything I have received in life.

What is your most marked characteristic?
Imagination.

Among your talents, which one(s) give(s) you a competitive edge?
I was not born with special talents, but I was born with a great deal of curiosity and a desire to develop new skills every day.

What do you consider your greatest achievement?
Every goal achieved is the biggest at the time. Every article achieved and published is the sum of the efforts of many people, so it is great.

If you could change one thing about yourself, what would it be?
I do not want to change anything. What I was and what I am have left me where I am now, and I feel proud of where I am, how I am, and who I am with.

What do you most value in your friends?
Appreciation and loyalty despite thousands of flaws and mistakes.

Who are your favorite writers?
Julio Cortazar, Horacio Quiroga, Jorge Luis Borges and Gabriel Garcia Marquez.

Who are your heroes of fiction?
None. My favorite is an antihero, the Joker, who reminds us of our dark humanity.

Who are your heroes in real life?
Anonymous people help others, but they do not appear in newspapers or social media.

What aphorism or motto best encapsulates your life philosophy?
"Stairs are climbed from the front, since climbing them from behind or the side will result particularly uncomfortable." — Julio Cortázar, "Instructions on How to Climb a Staircase" (from Cronopios and Famas, 1962). Trans. Paul Blackburn.

In the original: "Las escaleras se suben de frente, pues hacia atrás o de costado resultan particularmente incómodas." — Julio Cortázar, "Instrucciones para subir una escalera" (de Historias de cronopios y de famas, 1962).

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INNOVATORS & IDEAS: RESEARCH LEADER

Maria A. Oquendo: The translational pathway from the elucidation of the biological contributions to suicide risk to the development of interventions aimed at preventing morbidity and mortality

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Keywords: Suicide, suicidality, brain, population health, women in science

Dr. Maria Oquendo is Ruth Meltzer Professor and Chairman of Psychiatry at University of Pennsylvania and Psychiatrist-in-Chief at the Hospital of the University of Pennsylvania. A summa cum laude graduate of Tufts University, she attended College of Physicians and Surgeons, Columbia University and completed residency at Payne Whitney Clinic, New York Hospital, Cornell. She is a member of the National Academy of Medicine, one of the highest honors in medicine. Dr. Oquendo has used Positron Emission Tomography and Magnetic Resonance Imaging to map brain abnormalities in mood disorders and suicidal behavior. Her expertise ranges from psychopharmacology to Global Mental Health. She has over 500 peer-reviewed publications, an H-index 116 and 49,472 citations (Google Scholar). In terms of organizational leadership positions, Dr. Oquendo is Past President of the American Psychiatric Association (APA), the International Academy of Suicide Research, the American College of Neuropsychopharmacology (ACNP), and the American Foundation for Suicide Prevention's Board of Directors. She is Vice President of the College of International Neuropsychopharmacology and has served on the National Institute of Mental Health's Advisory Council. Dr. Oquendo serves on Tufts University's Board of Trustees, serves on its Executive Committee and chairs Tufts' Academic Affairs Committee. A recipient of multiple awards in the United States, Europe, and South America, most recently, she was honored with the Symonds Award (APA 2017), the APA's Research Award (2018), the Shockley Award (ACNP 2018), and the Glassman Award (Columbia University 2021). Dr. Oquendo has shared some of her thoughts and perspectives on her life and career.

The Genomic Press Interview Part 1: Maria Oquendo: Life and career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

I have loved mathematical concepts and numbers since middle school, but I also cherished language, art, and design. I originally thought that Architecture would help me meld these interests, yet the liberal college I attended did not offer such studies. Thus, I focused on theoretical math and Romance language literature. The rest of the trajectory to medicine is a yarn, but suffice it to say that at no time during my teens or twenties did I consider scientific inquiry my calling. I viewed science as key training to support pragmatic applications: architecture, medicine. And so, I finished residency in Psychiatry and chose a position as a teaching faculty member in a busy clinical service. It was not until I had been in that position for 8 years that I began to think about other opportunities. Fortuitously, one of my residency supervisors was recruited to Columbia University, where I was on the faculty. He was one of my research mentors during residency. A key point is that he knew I would work hard and offered me a full-time position on his team. I was not sure I would like the job. In fact, I thought there was a good chance I would hate it. But I was wrong. The experience was transformational. I loved thinking about how to interpret data, I loved statistics, I loved writing papers, carefully and methodically delineating the approach, the analysis, the results, and the conclusions. I even loved writing grants. I was in heaven. Even though I have had several administrative positions, it is undeniable that the core of my professional identity is as a scientist.

Figure 1. Maria A. Oquendo, MD, University of Pennsylvania, USA.

We would like to know more about your career trajectory leading up to your most relevant leadership role. What defining moments channeled you toward that leadership responsibility?

Many of my students and mentees have asked me how I forged the path to becoming Chairman of a major Department of Psychiatry. They are mostly taken aback when I tell them that I did not plan it at all and that I am as surprised as the next person that it happened. I also tell them that many leaders at my level have shared with me that their experience was not so different. It was not planned. It was the result of responding to opportunities even when doing so was not aligned with a personal vision of the trajectory. For example, when I shifted from a faculty position as a clinical educator to a research psychiatrist, I did not have any leadership responsibilities. However, within a few years, my mentor appointed me Director of the Clinical Lab for what was then the Division of Neuroscience in

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Psychiatry at Columbia. This was an opportunity I relished, and I did the job happily for 8 years until I was tapped by the Executive Vice-Chair of the Department of Psychiatry to become the Director of Ambulatory Research Clinics, of which there were 28 (!). I was far from sure that this administrative job was for me, but I applied the same methodical approach I used in research to understand what each clinic focused on and how it was organized. I wanted to know whether there was a need for a more uniform structure or whether the clinics used robust strategies in their management of clinical research patients in terms of safety, rigor, and productivity. I wanted to understand what their scientific output was and how each research clinic supported its efforts. Were they supported solely by state resources (after all, this was the New York State Psychiatric Institute which houses the Columbia University Department of Psychiatry) or did they have foundation or federal grants? Did they rely on philanthropy or did they use income from pharmacological trials or consultations? Suffice it to say that I learned a lot, but within one year, the Chairman of the Department tapped me to become Vice-Chairman for Education and Training Director. Here too, I was quite ambivalent about the role. I was concerned that it would be all-consuming and take me away from my science or worse, that it would bore me. Nonetheless, I decided to give it a whirl. This ended up being a key step in the journey towards Chairmanship. The Chairman of the Department also urged me to run for President of the American Psychiatric Association. Nothing could have been further from my mind. To me, such a step was misaligned with my goals and would probably drive me insane because of the politics, to boot. Soon, I had leaders from around the country calling and emailing asking me to run. My Chairman was insistent and after all, he was my boss. So, I went ahead. I was stunned to see how fun, yet difficult it was. It amused me no end that at international conferences, psychiatrists whom I had never met came up to me to have their picture taken with me. I was honored that so many people seemed to trust me to lead. It turned out this, too, was an important imprimatur for being considered "Chairman material." It should not have surprised me that high visibility married with academic chops was an excellent combination to be seen as a leader, but it did.

Please share with us what initially piqued your interest in your favorite area of research or professional focus
That the focus of my work has been on suicidal behavior was happenstance. I was not looking to do research per se, but when my mentor approached me to work in a clinical research lab, I said my interest was in depression and cross-cultural issues related to it. I started off with that, but soon gravitated towards the mainstream work of the lab because that is where most of the biological focus was. It was clear to me that biological work was the most highly valued in that lab. I learned about positron emission tomography, cerebrospinal fluid studies, postmortem brain studies enough that I could conduct some of the statistics and interpret the data. It turned out that neurobiology was some of the most interesting part of the work to me.

What kind of impact do you hope to achieve in your field through your focus on your specific research topics?
I hope to raise scientific awareness of the biological contributions to suicide risk which can translate to interventions to prevent morbidity and mortality. I also hope to decrease clinician's anxiety about managing suicidal patients using implementation science strategies.

Could you tell us more about your current scholarly focal points within your chosen field of science?
I have been working on delineating the risk for suicidal ideation and behavior among persons who do not meet the criteria for psychiatric disorders. As a departing point, I have focused on raising scientific awareness about the frequency with which suicidal behavior occurs unaccompanied by other psychiatric morbidities. When I first started writing up the data, I was stunned by how married the field was/is to the notion that suicidal behavior only rarely happens absent at least one psychiatric disorder. Data documenting the contrary appeared in publications but went unmentioned in discussions, never mind the titles of articles. The prevailing clinical lore is that if suicidal behavior occurs without mental illness, it must be because the disorder is "masked." The data that contradicts that notion abounds and requires scientific attention for what it does: defy our current clinical wisdom.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences, that you uphold within your own research environment?
Attention to detail, internal logic in formulating research studies and writing manuscripts, and lucidity and linearity in writing.

At Genomic Press, we prioritize fostering research endeavors based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that you think warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
Many people talk about this, but to me, the metrics to measure productivity and quality in research are heavily biased toward western scientists' work and in medicine, toward basic science. We must do better.

What do you most enjoy in your capacity as an academic and research leader?
I love discussing ideas for experiments and studies as well as writing grants and manuscripts. As a Chair, I enjoy encouraging faculty who don't usually work together to collaborate on an important research opportunity and seeing what they come up with.

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
I very much enjoy traveling and appreciate diversity in what I do. In some cases, the goal is to enjoy cultural aspects of the location (history, local culture, art, music, architecture), but at other times it is more about the gastronomy of the place, and not necessarily in fancy establishments. I also thoroughly enjoy nature and am an avid hiker although not a mountaineer, by any stretch! For me, it is about being outside, exploring habitats with their flora and fauna, and soaking in vistas with a day pack and my recently acquired, and immediately beloved, walking sticks for more challenging treks.

The Genomic Press Interview Part 2: Maria Oquendo: Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
A day at the beach, sitting in the shade, watching the waves roll in.

What is your greatest fear?
Losing my memory.

Which living person do you most admire?
Sonia Sotomayor.

[1] In the late 19th century various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. Multiple other historical and contemporary figures have answered the Proust Questionnaire, such as Oscar Wilde, Karl Marx, Arthur Conan Doyle, Stéphane Mallarmé, Paul Cézanne, Martin Boucher, Hugh Jackman, David Bowie, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

What is your greatest extravagance?
I love jewelry. I buy it sparingly and carefully. It is an interest that I shared joyfully with my late mother who bought me jewelry as a youngster. I relished going to jewelry shops with her as she aged and buying her lovely pieces that captured her fancy.

What are you most proud of?
My sons are kind, compassionate, considerate, hardworking, wonderful people.

What is your greatest regret?
At the risk of sounding glib, not doing a junior year abroad in college.

What is the quality you most admire in people?
In Spanish, one can describe a person as noble. It has nothing to do with lineage. It is about kindness, morality, and compassion.

What do you consider the most overrated virtue?
Carefreeness.

What is your favorite occupation?
Architecture.

Where would you most like to live?
I am hoping to spend a year living in Spain, my country of origin, in the next years. Although it would ideally be in Barcelona, there are many wonderful places in Spain I would love to call home.

What is your most treasured possession?
By far, my sense of humor.

When and where were you happiest? And why were so happy then?
I would say that with each passing year, I feel happier. I think that the wisdom that accrues brings peace and perspective. Even though many things decline with age, the accrual of wisdom overshadows those losses.

What is your most marked characteristic?
A propensity for raucous laughter.

Among your talents, which one gives you a competitive edge?
A natural inclination to tell people what they do well or about positive things I have heard about them.

What do you consider your greatest achievement?
Election to the National Academy of Medicine.

If you could change one thing about yourself, what would it be?
My tendency to angst about the future.

What do you most value in your friends?
Integrity, trustworthiness, intelligence, humor, and warmth.

Who are your favorite writers?
Favorite current writers include Jill Lepore and John McPhee. Also, Gabriel Garcia Marquez, Alejo Carpentier, Julio Cortazar, and Jorge Amado.

Who is your hero of fiction?
I don't tend to think that way. Everyone has foibles.

Who are your heroes in real life?
As above.

What aphorism or motto best encapsulates your life philosophy?
Do the right thing. To which I would add, "timely."

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INNOVATORS & IDEAS: RESEARCH LEADER

Gustavo Turecki: Three fundamental questions – How does the brain respond to social and emotional experiences? Why does psychological trauma trigger depressive states? What are the mechanisms of antidepressant responses?

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Keywords: Major depressive disorder, treatment-resistant depression, suicide, trauma, antidepressant response

Gustavo Turecki MD PhD FRSC is a clinician scientist whose work focuses on understanding brain molecular changes that occur in major depressive disorder and suicide, as well as molecular processes that explain antidepressant treatment response. Dr. Turecki is Full Professor and Chair of the Department of Psychiatry at McGill University, the Scientific Director and Psychiatrist-in-Chief of the Douglas Institute in Montreal, Canada, where he also heads the Depressive Disorders Program. He has authored over 600 publications, including research articles in leading peer-reviewed journals such as *Nature Neuroscience, Nature Medicine*, and *The Lancet* and is among the world's most highly cited scientists according to Clarivate, Web of Science. He has received several national and international awards and sits on several advisory boards. Dr. Turecki graciously offers our audience a glimpse into his personal and professional journey.

The Genomic Press Interview Part 1: Gustavo Turecki – Life and career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

Since my childhood, I have been fascinated with science and medicine. When I got into medical school, I soon became interested in physiology of exercise. Having been a competitive swimmer, this seemed like a natural extension of my previous interests. I was ready to work in sports medicine until I did my rotation in psychiatry. Unexpectedly, I found myself fully fascinated by this field; therefore, after some internal debate and ambivalence, I decided to pursue this specialty. Early in my psychiatry residency, I was involved in a case of dizygotic twins that strongly influenced my professional trajectory and research career. I was also fortunate to count with excellent role models early on. They were instrumental in my professional development, providing me with excellent advice and, above all, they instilled in me core professional, scientific and personal values that have been essential, as I pursued a career in academic medicine.

We are keen to explore your career trajectory leading up to your most relevant leadership role. What defining moments channeled you toward that leadership responsibility?

Although today I hold several leadership roles, leadership did not come naturally to me. I remained in academia because of the research work and the intellectual stimulation that it provides, not to be a manager. I first took a leadership role out of duty, but it was difficult as leadership involves skills that I had to acquire with effort. In addition, I was very concerned about the potential impact that the time I had to dedicate would have on my lab and research. After many years in diverse leadership roles, I now appreciate the opportunity that leadership provides, and particularly, the opportunity to build capacity and contribute to develop academic psychiatry, research and clinical services.

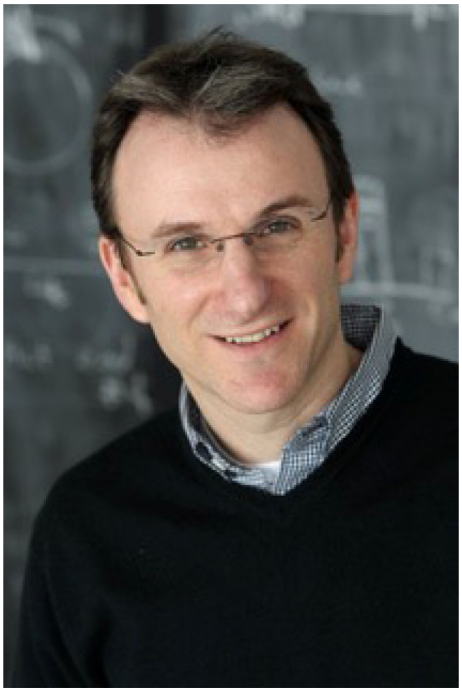

Figure 1. Gustavo Turecki, MD, PhD, McGill University, Canada.

Please share with us what initially piqued your interest in your favorite area of research or professional focus.

As a resident, I treated one of a dizygotic twin who had a shared delusion with her co-twin. The case was fascinating and led me to explore, conceptually, the role of genetics in the etiology of mental illness. I have been working in genetics and genomics ever since.

What kind of impact do you hope to achieve in your field through your focus on your specific research topics?

Above all, I hope my work will contribute to elucidate processes and mechanisms underlying psychopathology, and particularly major depressive disorder and suicide risk, which are my areas of more direct interest. More specifically, I hope my work will help gain some insight into how the brain responds to social and emotional experience and how traumatic experiences trigger pathological depressive states. I also hope that my work may help elucidate mechanisms of antidepressant response.

I keep a clinical practice, specializing in refractory or treatment-resistant major depressive disorder. It is extremely rewarding to help people who suffer and are unable to function. While the treatments we use

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today are generally effective, they do not always work, and sometimes, it takes way too long to identify the proper treatment or for the treatment to work effectively. I hope the work that I do will eventually help the life of people like the patients I treat.

Could you let us know your current scholarly focal points within your chosen field of science?
Currently, I am interested in the understanding of molecular changes associated with depression at single-cell resolution. We have adapted diverse single-cell genomic methods to study postmortem human brain tissue and are exploring different aspects of major depression. We are also very interested in the role of extra-cellular vesicles in systemic communication and how their cargo may be manipulated to elicit therapeutic responses.

What do you most enjoy in your capacity as an academic and research leader?
The intellectual stimulation of the work and the possibility of contributing to knowledge.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences, that you uphold within your own research environment?
Research is stressful and competitive, but I believe that the lab environment should be welcoming and supportive, and very collegial.

At Genomic Press, we prioritize fostering research endeavors based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that you think warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
I am passionate about science and the work that I do, and this is what drives me and has been a constant motivation throughout my professional trajectory

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
I have many personal interests. Besides my family life, I keep busy and try to live a balanced life. I am physically active, exercising almost daily. I enjoy skiing, biking, cooking and good wine. I grow a vegetable garden in the summer, and love politics. I am an avid reader of the economist and diverse newspapers, and a regular listener of the Good Fight by Yascha Mounk and several other podcasts.

The Genomic Press Interview Part 2: Gustavo Turecki – Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
All moments of happiness are just perfect.

What is your greatest fear?
Decline.

Which living person do you most admire?
Too many to list.

What is your greatest extravagance?
A bottle of Gran Enemigo Gualtallary.

What are you most proud of?
My three kids.

What is your greatest regret?
None.

What is the quality you most admire in people?
Their intellect.

What do you consider the most overrated virtue?
Virtues are virtues, can't be overrated.

What is your favorite activity (physical or intellectual)?
Too many to list.

Where would you most like to live?
Right where I live, in Montréal.

What is your most treasured possession?
Possessions come and go, so I do not treasure them. They are worth for their transaction value.

When and where were you happiest? And why were so happy then?
Right now, yesterday and tomorrow.

What is your most marked characteristic?
Not sure I can answer this, but probably persistence.

Among your talents, which one do you think gives you a competitive edge?
Perhaps the fact that I am patient, but very persistent.

What is a personality/characteristic trait you wish you had?
To be more extroverted.

What do you consider your greatest achievement?
Scientifically, it was the first description of how early-life adversity leads to molecular changes in the brain through epigenetic changes (McGowan et al, 2009).

What do you most value in your friends?
Their sense of humor.

Who are your favorite writers?
Chabon, Singer, Borges, Amis, Joshua Cohen, Richler, Cortazar, Philip Roth, Bioy Casares, Beauvoir, Atwood, Amos Oz, and several others.

Who are your heroes of fiction?
Many, but to cite a recent one, Gyuri Köves in Fatelessness by Imre Kertész, which I have just read.

Who are your heroes in real life?
My grandparents, who escaped Nazi occupied East Europe and established in South America after losing many of their family members and much hardship. They had nothing, worked very hard, kept going, appreciated everything they had and were always in a good mood. They have a been a constant source of inspiration.

What aphorism or motto best encapsulates your life philosophy?
You make the best of every situation.

[1] In the late nineteenth century various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. Multiple other historical and contemporary figures have answered the Proust Questionnaire, such as Oscar Wilde, Karl Marx, Arthur Conan Doyle, Stéphane Mallarmé, Paul Cézanne, Martin Boucher, Hugh Jackman, David Bowie, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

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Edo Ronald de Kloet: How does the action of glucocorticoids change from protective to harmful? What is the cause? And what are the consequences?

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Keywords: Psychoneuroendocrinology, pharmacology, stress, brain, cortisol, stress-related disease

For half a century, Edo Ronald (Ron) de Kloet has pursued a fundamental question in neuroscience: how do stress hormones switch from protecting our brain to potentially harming it? After receiving his PhD in 1972 at the University of Utrecht under David de Wied's mentorship, he spent two formative years with Bruce McEwen at Rockefeller University before returning to the Rudolf Magnus Institute. In 1990, he was appointed Professor of Medical Pharmacology at Leiden University, where he discovered how a single hormone – cortisol – could protect and damage the brain through two distinct receptor systems (MR and GR). This finding opened new paths for understanding and treating stress-related mental disorders. His research, spanning over 600 publications, has transformed our grasp of how the brain copes with stress and earned him numerous honours, including the Geoffrey Harris Award (2005), the ECNP Award (2007), and the Golden Emil Kraepelin Medal (2014) for advancing our understanding of depression. Though officially "retired" since 2009, he remains active as an emeritus professor at Leiden University Medical Centre and academy professor at the Royal Netherlands Academy of Arts and Sciences. Recognizing his contributions to science and society, he was knighted in the Order of the Dutch Lion in 2010. Recently, alongside his long-time collaborator Professor Marian Joëls, he received the 2024 Global Stress & Resilience Network Pioneer Award. In this Genomic Press Interview, Dr. de Kloet reflects on his remarkable journey and shares fresh insights into the fascinating world of stress neuroscience.

Part 1: Ron de Kloet – Life and Career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

My two older brothers obtained a PhD in biochemistry and molecular biology. During their thesis research, I sometimes joined them in the lab, which inspired me to enter a biochemistry program at the University of Utrecht in 1961. After a dull Bachelor's, I became excited when starting my Master's hands-on research by isolating novel bioactive peptides from the sheep pineal gland, even more so during my Endocrinology training at Organon Pharmaceuticals. I learned from Professor Marius Tausk, the director, that "Endocrinology is a concept, an approach, or even a method. Whatever the specific endocrine subdiscipline, topic, or subject, the binding element is the objective: understanding how signals coordinate the processes in cells, tissues, and organs." This sophisticated view of endocrinology intrigued me and a student colleague at Organon so much that we both wanted to apply for an available neuroendocrinology PhD position guided by the famous Professor David de Wied. However, since we did not want to compete, we did it by flipping a coin. My colleague won and got the position, and I was left empty-handed!

Figure 1. Edo Ronald de Kloet, PhD, Leiden University Medical Centre, The Netherlands.

A month later, I arranged an appointment with Professor de Wied. I related the coin-flipping story on Thursday, 28 November 1968, at 9.00 am. Then, after some discussion, Professor de Wied said: "I'll call the Director of Organon." After 5 minutes, the call ended, and De Wied said, "You can start this coming Monday, 2 December, at Organon with a PhD project." He also defined the topic of my thesis. Bruce McEwen had just published his hallmark paper on the retention of tracer amounts of ^{3}H-corticosterone in cell nuclei of the hippocampal pyramidal and dentate gyrus neurons. De Wied said, "We can do this better, Ron! We will examine the central action of the much more potent glucocorticoid dexamethasone."

After two years, I wrote Bruce a letter stating that I could not reproduce his finding of corticosterone binding in the hippocampus with

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dexamethasone, and he invited me over to New York to solve the issue in his lab. As a postdoc in Bruce's lab, we confirmed the inability of dexamethasone to label the corticosterone receptors in the hippocampus. Only 20 years later, using mouse mutants obtained from Piet Borst, we found out why: Dexamethasone, rather than corticosterone, is a substrate for multidrug resistance P-glycoprotein (mdr-Pgp) in the blood-brain barrier, which pumps the synthetic steroid out of the brain! Instead, dexamethasone acts in the anterior pituitary corticotrophs to suppress stress-induced ACTH release, a fundamental finding for understanding the Dexamethasone Suppression Test, a laboratory test assisting in the diagnosis of depression, further developed into the Dex/CRH test by my colleague Florian Holsboer in Munich.

I learned that (i) luck requires a prepared mind, (ii) partial reinforcement extinction works, and (iii) one needs patience.

We would like to know more about your career trajectory leading up to your most relevant leadership role. What defining moments channelled you toward that leadership responsibility?

The second phase in my career started with my tenured appointment in 1975 as Associate Professor at the Rudolf Magnus Institute under De Wied's guidance upon returning from Rockefeller University. The staff position required the development of a neuropharmacology teaching program in biomedical sciences and participation in the Institute's research on neuropeptides. The term' neuropeptides' was coined in the late sixties by David de Wied to define the central effects of fragments of vasopressin, oxytocin, and ACTH that were devoid of their classical endocrine activity. For instance, the fragment vasopressin (4–9), the "memory pill", reinforced memory consolidation of fear-motivated behaviour.

The late '70s were exciting times for neuropeptides! With Eva Mezey and Dan Dorsa, we showed that peptides cleaved from pituitary hormones could reach the brain via retrograde transport in the pituitary stalk and the perivascular space. With Peter Burbach, we identified the brain endopeptidases that generated vasopressin, oxytocin, and ACTH-derived neuropeptides from larger precursor molecules. Miklos Palkovits taught me in the mid-70s neuro-anatomy and the ability to punch more than 100 different nuclei from frozen brain sections (600 punches/hour), serving many other research groups.

In 1984, with Anat Biegon, Door Voorhuis, and Jack Elands, we discovered the distribution of oxytocin and vasopressin receptors in discrete rat brain regions using in vitro autoradiography. That discovery culminated in an exciting twist in the songbird: testosterone-induced vasotocin receptors were concentrated around a song nucleus in the canary brain (n. robustus archistriatalis). Stimulation of these receptors modulated the development of the stereotyped canary song.

I learned that to grow toward a leadership role in neuroscience, you need to collaborate with experts in research on various layers of biological organization, from molecules to cells and circuits to behaviour. While the above experiences were exciting, the real breakthrough toward leadership was understanding how glucocorticoids act, as detailed in the next section.

Please share with us what initially piqued your interest in your favourite research or professional focus area.

In 1985, we had a "Eureka" moment in recognizing the identity of the rodent hippocampal corticosterone receptors. At that time, Roussel Uclaf had synthesized a 'pure' glucocorticoid, distinguishing between mineralocorticoid receptors (MR) that bind corticosterone with a 10-fold higher affinity than the classical glucocorticoid receptors (GR). With Dick Veldhuis and Hans Reul, we realized that the tracer doses of ^{3}H-corticosterone provided a sufficient amount to occupy the MR but not the GR. For GR occupancy, corticosterone concentrations must increase to levels circulating around the circadian peak or after stress. With Hans Reul, Anke van Eekelen, and Win Sutanto, we published the distribution of MR and GR in the rat brain using in vitro autoradiography, immunocytochemistry, and in situ hybridization. With Chris Edwards from Edinburgh, we demonstrated that the enzymatic breakdown of naturally occurring glucocorticoids was essential for the MR to become aldosterone-specific in epithelial cells such as the kidney.

With an understanding of the complementary MR- and GR-mediated actions of corticosterone and cortisol, we mined gold. Suddenly, we knew how to design experiments that made biological sense in stress research-the Eureka moment triggered an avalanche of studies. It provided the inroad to the group's transition to Leiden University in 1990, the Division of Medical Pharmacology, with my promotion to full Professor at the Leiden/Amsterdam Centre of Drug Research (Leader Douwe Breimer). In neurophysiology, we had an intense collaboration with Professor Marian Joëls at the University of Amsterdam, who discovered MR and GR's complementary role in regulating transmitter responses and ion conductances in a U-shaped relationship: MR activation transiently increased hippocampal excitability, which was suppressed, subsequently, by stress-induced GR activation. Marian Joëls and Henk Karst discovered in 2005 that MR can also mediate rapid non-genomic actions in the hippocampus.

With Anna Ratka, we showed the physiological role: MR activation is essential for the tone and activation of the stress response system, while subsequent GR activation facilitates suppression by negative feedback. In behaviour, Melly Oitzl found that MR is necessary for retrieving information and selecting a coping style, while GR activation promotes memory consolidation. Menno Kruk made a case for MR-dependent anxiety/aggression-driven phenotypes. Nicole Datson pioneered gene expression profiling in laser-dissected brain regions and identified numerous novel glucocorticoid-responsive pathways in the brain under stress, particularly in epigenetic processes. Erno Vreugdenhil discovered a glucocorticoid-responsive neuroplasticity gene doublecortin-like kinase, which functions in microtubules during development and neurogenesis. With Seymour Levine, we made significant contributions to the role of glucocorticoids in programming stress-coping and adaptation in neonates for later life.

Thus, a 1968 PhD project developed into a successful research program. I learned that focus, collaboration, and mutual respect are the ingredients for an exciting scientific journey. Melly, Menno, Erno, Nicole, Onno Meijer, and Roel de Rijk formed, as group leaders, a dream team, guiding together more than 200 Master's and PhD students, postdocs, guests, and technical and administrative staff, each delivering a unique contribution that I cannot highlight due to space limitations. See for a summary of the first 30 years of my career in David de Wied's Festschrift: de Kloet ER. Stress in the brain. Eur J Pharmacol. 2000;405:187–198.

What impact do you hope to achieve in your field by focusing on specific research topics

2009 I reached emeritus status, but I had enough grant money to continue for five years before the Dutch mandatory retirement at 70.

Collectively, we have contributed to the knowledge of how cortisol coordinates body and brain function to support coping and adaptation and how the hormone programs this adaptive response for life. We formulated the MR: GR (im)balance hypothesis: "Upon imbalance of the MR and GR-mediated actions, the stress response's initiation and management becomes compromised. At a certain threshold, this may lead to a condition of neuroendocrine dysregulation and impaired behavioural adaptation, which potentially can aggravate stress-related deterioration and promote vulnerability."

We collected data (and are still doing so) to test this hypothesis. Two out of several highlights. Firstly, Roel de Rijk and Liane Klok discovered an MR haplotype associated with optimism that protects against depression. This discovery earned US and EU patents as signs of a successful translational opportunity. Secondly, Onno Meijer became my successor in Leiden and continued to work on glucocorticoids developing, with the support of Corcept Therapeutics, novel Selective Glucocorticoid / Mineralocorticoid Receptor Modulators (SGRM / SMRM) targeting tissue selective receptor co-regulators. With this prospect, glucocorticoid therapy will have fewer side effects.

Please tell us more about your current scholarly focal points within your chosen field of science.

I was fortunate to participate in the exciting projects of Alex de Nicola's group (Buenos Aires), which has been pioneering the striking ability of SGRM and SMRM to reverse neuropathology in animal models of chronic

Figure 2. Ron de Kloet sailing Nordic Folkboat VoiVoi.

stress, hypertension, and neurodegenerative diseases. Some of these Corcept compounds are now in phases 2 and 3.

I am happy to participate in a challenging program led by Megan Galbally (Melbourne, Australia), who explored the prevalence of childhood anxiety disorder in the offspring of mothers from the Mercy Pregnancy Emotional Wellbeing Study, a longitudinal cohort study of pregnant women exploring the impact of perinatal depression (from conception to birth) at delivery, and 6 months, 12 months, and 4 years postpartum. The outcome of these exciting studies aligns with developmental animal studies: stress during early life programs via cortisol action in the amygdala emotional reactivity for later life.

I occasionally write a commentary or review on stress. For instance, with Marc Molendijk, we wrote a series of articles on anthropomorphism in neuropharmacology, using the forced swim test as an example.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences that you uphold within your research environment?
Examining a fundamental neuroscientific question requires a multidisciplinary approach in a social, behavioural, biochemical/molecular, and physiological context. Keeping up team spirit requires frequent meetings to discuss progress. Team spirit and social lab life go together: joint coffee breaks, lunches, sports, and 'cabaret' are essential for shaping collegial trust and passion.

At Genomic Press, we prioritize fostering research endeavours based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
I support the European 'Agreement on Reforming Research Assessment'. This agreement between the European Commission, Science Europe, and the European University Association (EUA) endorses the objectives of our National Recognition & Rewards program. The agreement focuses on recognizing and rewarding academics for their various tasks. This focus implies that research assessment primarily occurs through a qualitative evaluation rather than based on the number of publications, h-indices of authors, or journal impact factors. See https://recognitionrewards.nl/2022/10/10/dutch-knowledge-institutions-sign-european-agreement-on-reforming-research-assessment/.

What do you most enjoy in your capacity as an academic or research leader?
To inspire students about the beauty of the brain, from genes to behaviour, in all imaginable contexts, discuss in-depth new findings, and guide young scientists in the first years of their scientific life.

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
I have a semi-professional online weather station called 'The Hippocampus,' in a polder 4 m below sea level close to where I live. Just a few highlights: I could measure the air pressure changes and wind shifts following the January 2022 Pacific Hunga Tonga-Hunga Ha'apai submarine volcano outburst. I like ice skating, swimming, sailing, hiking, and gardening.

Part 2: Ron de Kloet – Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
I realize that happiness is context-dependent, and here are some ingredients. During exercise, my endorphins work; during social life, oxytocin may peak; if I compete, dopamine helps to pursue success; and serotonin gives a sense of control during stress. It is all perfect if it works.

What is your greatest fear?
The feeling that I have no control.

Which living person do you most admire?
I admire my brother for saying, "Doing nothing is not an option," and he succeeded in extending the high-quality life of one of his beloved sons for another 8 years.

What is your greatest extravagance?
To keep my 65-year-old mahogany wooden Nordic Folkboat in excellent shape.

What are you most proud of?
In my scientific life, I am most proud of my 57 PhD students, who all successfully defended their thesis.

What is your greatest regret?
I should have reserved more time for social activities and reading.

[1]In the late nineteenth century, various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. In 2003 Proust's handwritten answers were auctioned off for $130,000. Multiple other historical and contemporary figures have answered the Proust Questionnaire, including among others Karl Marx, Oscar Wilde, Arthur Conan Doyle, Fernando Pessoa, Stéphane Mallarmé, Paul Cézanne, Vladimir Nabokov, Kazuo Ishiguro, Catherine Deneuve, Sophia Loren, Gina Lollobrigida, Gloria Steinem, Pelé, Valentino, Yoko Ono, Elton John, Martin Scorsese, Pedro Almodóvar, Richard Branson, Jimmy Carter, David Chang, Spike Lee, Hugh Jackman, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions, an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

What is the quality you most admire in people?
Sense of humour, reliability.

What is the trait you most dislike in people?
Narcissism

What do you consider the most overrated virtue?
In science, when an individual's h-index is a criterion for judging scientific quality and a predictor of future performance and success.

What is your favourite activity?
A daily hike of an hour is inspiring and gives a good feeling.

Where would you most like to live?
Rural environment, at a lakefront, with Dutch weather.

What is your most treasured possession?
Science-related: my 1000+ hippocampus items.

When and where were you happiest? And why were so happy then?
Again, context-dependent. An example: February 1996, Wengen, Switzerland. We rented a cottage and went skiing and hiking; the weather was fantastic, and there was a silent and impressive red evening sun on the 4000m high mountains. Alternatively, sailing or wandering with Marian through nature, or understanding experimental data.

What is your current state of mind?
Quiet, but with a sense of urgency for things to do

What is your most marked characteristic?
Interest in the other person, what they do, how they think.

Among your talents, which one(s) give(s) you a competitive edge?
In science, to identify talent that can synergize in a multidisciplinary fashion to reach a common goal.

What do you consider your greatest achievement?
With my associates' help, we have provided evidence to substantiate that cortisol action controls a switch between resilience and vulnerability in adaptation to chronic stress.

If you could change one thing about yourself, what would it be?
Be more creative in thinking out of the box.

What do you most value in your friends?
Integrity.

Who are your favourite writers?
John Grisham's detectives, Marten Toonder's 177 Bommel stories, Val Howells's 'Sailing into Solitude,' and Marian Joëls' latest book, "Finished," (ResearchgGate), a novel about the world of science, confronting and written with compassion and wit.

Who are your heroes of fiction?
Kwetal, a subterranean dweller and mastermind, is my hero in the 38th story from the Bommelsaga, written and drawn by Marten Toonder. Kwetal coined the term "Denkraam" (no English translation) to describe the brain.

Who are your heroes in real life?
Herman van Praag (1929–). I attended the presentation of his latest book (November 2024) a few days before this interview. Entitled: Gemoedsbewegingen (emotional movements). Professor Herman van Praag founded Biological Psychiatry in the Netherlands. My other heroes passed away.

What aphorism or motto best encapsulates your life philosophy?
"As 't net kin sa't moat, dan moat 't mar sa't kin". It is from the Frisian language, where my roots are. In English, it would be something like: "If it cannot be done as it should be, then it should be done as it can."

Leiden, The Netherlands
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Genomic Psychiatry

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INNOVATORS & IDEAS: RESEARCH LEADER

Mayana Zatz: Two critical questions take center stage – Which variants mitigate the impact of lethal mutations in severe conditions with mild phenotype? What factors contribute to the health and longevity of centenarians?

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Keywords: Neuromuscular disorders, protective variants, aging, genome studies, genetic counseling, ethics

Mayana Zatz has been a Professor of Genetics at the Institute of Biosciences, University of São Paulo (USP), Brazil, since 1982. She became an assistant professor after a postdoc at the University of São Paulo and a second postdoc at the University of California, Los Angeles. Her current research is focused on neuromuscular disorders, aging, genomics, and, more recently, xenotransplantation and the use of the Zika virus as an oncolytic therapy against brain tumors. Functional studies are done in genetically engineered mouse and cell models. She is particularly interested in investigating protective mechanisms in rare patients with Duchenne dystrophy and a milder clinical course, as well as in centenarians' health determinants. Mayana Zatz is also involved in ethical aspects of genomic studies and government political decisions related to science. Professor Zatz is pleased to offer our readers insights into her personal and professional experiences.

Part 1: Mayana Zatz – Life and Career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

I have been fascinated with science for as long as I can remember, and I loved reading the biographies of famous scientists such as Madame Curie or Pasteur. In high school, I fell in love with genetics. It was in the pre-molecular era, but I was intrigued by how genetic traits were transmitted across generations. I decided to pursue these studies in my adult life.

We would like to know more about your career trajectory leading up to your most relevant leadership role. What defining moments channeled you toward that leadership responsibility?

When I started to study genetics, I did not imagine I would be a leader. My ambition was to pursue a career as a geneticist at the University of São Paulo, which was and still is the best Brazilian University. After returning from my postdoc at the University of California, Los Angeles, I submitted a small grant to FAPESP (São Paulo Research Foundation, in Portuguese: Fundação de Amparo à Pesquisa do Estado de São Paulo), our leading research funding agency, to continue my research on muscular dystrophies. DNA technology was unavailable; therefore, my studies used serum enzymes to investigate different forms of muscular dystrophy. I started forming a group of young students, primarily undergraduates, interested in this subject. One day, I was invited to a meeting at FAPESP, and I learned that only scientific leaders had been invited. That was when I realized that I was considered a leader and that I had a greater responsibility.

Please share with us what initially piqued your interest in your favorite research or professional focus area.

My initial interest was muscular dystrophies. A turning point in my career was when a young woman who had three nephews affected by Duchenne dystrophy came to me for advice. She was getting married and worried about the possibility of having affected sons. At that time, nobody was working with muscular dystrophies in Brazil, and it attracted my interest. I wanted to understand the clinical variability among different forms of muscular dystrophies and the underlying genetic mechanisms. I also aimed to estimate the genetic risks for healthy female relatives to have affected sons. With my colleagues, Maria Rita Passos Bueno and Mariz Vainzof, we identified several novel genes responsible for neuromuscular disorders. Later, we discovered that patients with the same pathogenic mutation could have a highly variable course, showing that other factors could modulate the phenotype. Since then, my research has focused on studying protective genetic variants in sporadic patients with Duchenne muscular dystrophy who are not as weak as one would expect from examining their genotype. We are also generating mice models carrying candidate modifier variants. Understanding the underlying "protective" mechanisms could open new avenues for treatment. More recently, my research focus has been on healthy centenarians. We are doing functional studies with IPS-derived cell lines from these centenarians. One intriguing question is whether they have genetic variants similar to top athletes.

Figure 1. Mayana Zatz, PhD, University of São Paulo, Brazil.

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Published online: 21 February 2024.

What impact do you hope to achieve in your field by focusing on specific research topics?
I hope to find novel therapies for muscular dystrophies if we can understand the protective mechanisms against the effects of pathogenic mutations. In the case of centenarians, we are also trying to understand the role of protective aging variants and whether their product could help promote healthy aging for people who were not born with these protective variants. I decided to focus on centenarians because it is known that genetics plays a significant role in older people's resilience, particularly after the age of 90.

Please tell us more about your current scholarly focal points within your chosen field of science.
I am coordinating several projects on the subjects I just described: Duchenne muscular dystrophy, centenarians, genomics, and ethics. I am also involved in two other projects: xenotransplantation (aiming to use genetically modified pigs as organ donors), and using the Zika virus as an oncolytic vector against brain tumors.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences that you uphold within your research environment?
In my research environment in Brazil, one must be prepared to deal with much bureaucracy; therefore, you need to manage frustration and be resilient. However, in São Paulo, where I live, we have an excellent research funding agency, FAPESP. Therefore, we are in a much better situation than scientists from other Brazilian states. I believe that contact with patients is precious for enhancing research motivation. Knowing the story behind the sample you are working with and the hope patients put into your research gives you a tremendous sense of responsibility. You know that you have to try your best.

At Genomic Press, we prioritize fostering research endeavors based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
In Brazil, the main challenge is to increase funding for science. Another significant challenge is to have a dramatic cost reduction in newly developed treatments for rare diseases, such as spinal muscular atrophy, hemophilia, or sickle cell disease, in order to make them available to all patients.

What do you most enjoy in your capacity as an academic and research leader?
Being a scientist is fascinating. When you understand a question, you open many others, and it is like playing an endless puzzle. You never get bored because what drives our motivation are the questions. What moves us as scientists is our tremendous curiosity. I love to discuss ideas with my students or try to solve problems while jogging in the morning. Also, I love it when young students approach me and say that they decided to be scientists because of my influence.

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
I like to read primarily biographies of interesting people; I love traveling and good movies. I love to be with my family and friends.

Part 2: Mayana Zatz – Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
I do not believe in perfect happiness. We have moments when we are delighted and others when we may be sad.

What is your greatest fear?
To lose my independence or cognitive capacity with aging.

Figure 2. Mayana Zatz and Laura, a 104-year-old swimming champion.

Which living person do you most admire?
I hold great admiration for several pioneers in genetics, notably Shinya Yamanaka, Emmanuelle Charpentier, and Jennifer Doudna.

What is your greatest extravagance?
Spending on traveling.

What are you most proud of?
My children and grandchildren and also some of my former students who became great scientists.

What is your greatest regret?
Not having more children.

What is the quality you most admire in people?
Honesty and courage.

What do you consider the most overrated virtue?
Modesty.

What is your favorite occupation (or activity)?
Scientific research.

[1]In the late nineteenth century, various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. Multiple other historical and contemporary figures have answered the Proust Questionnaire, such as Oscar Wilde, Karl Marx, Arthur Conan Doyle, Stéphane Mallarmé, Paul Cézanne, Martin Boucher, Hugh Jackman, David Bowie, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions, an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

Where would you most like to live?
I love the place where I live now.

What is your most treasured possession?
My house.

When and where were you happiest? And why were you so happy then?
When my daughter was born. I wanted to have a girl as I already had a son. And right before she was born, I finished writing my PhD thesis and moved to the house where I now live. I remember that coming from the hospital with her into my new house was the happiest moment in my life.

What is your most marked characteristic?
I am incredibly driven.

Among your talents, which one(s) gives you a competitive edge?
Creativity, not giving up quickly, and not being afraid to test new ideas.

What do you consider your greatest achievement?
My scientific career.

If you could change one thing about yourself, what would it be?
My age.

What do you most value in your friends?
Sincerity.

Who are your favorite writers?
George Orwell (1984) and Walter Isaacson (biographies).

Who are your heroes of fiction?
Forrest Gump.

Who are your heroes in real life?
My greatest hero was Nobel laureate Rita Levi-Montalcini, who died at age 103 while still active.

What aphorism or motto best encapsulates your life philosophy?
Never believe that you have achieved the best. There is always room for improvement.

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INNOVATORS & IDEAS: RESEARCH LEADER

Noboru Hiroi: Exploring the cellular and developmental origins of neuropsychiatric disorders linked to human copy-number variation

Genomic Psychiatry January 2025;1(1):21–23; doi: https://doi.org/10.61373/gp024k.0013

Keywords: 22q11.2, autism, cell model, copy number variation, intellectual disability, mouse model, schizophrenia

Professor Noboru Hiroi is a faculty member in the Departments of Pharmacology, Cellular and Integrative Physiology, and Cell Systems & Anatomy at the University of Texas Health Science Center at San Antonio (UT Health San Antonio), USA. He joined his current institution in 2019 after working at Albert Einstein College of Medicine in New York for 21 years. His current work is focused on the cellular and developmental origins of the dimensions of neuropsychiatric disorders in genetically engineered mouse and cell models. Professor Hiroi is happy to provide our readers with reflections on his life and career.

Part 1: Noboru Hiroi – Life and Career

Could you give us a glimpse into your personal history, emphasizing the pivotal moments that first kindled your passion for science?

I was born in a small town near the port city of Yokohama, Japan. It is surrounded by mountains and the ocean, and there is rich animal life. I was always fascinated by the normal and abnormal ways animals behave. As an undergraduate student at Waseda University in Tokyo, a lecturer who spent his sabbatical year at McGill University (Montreal, Quebec, Canada) taught me about the discoveries by James Olds and Peter Milner of a reward center in the rodent brain, D.O. Hebb's conceptualization of synaptic plasticity, and multiple memory systems by Brenda Milner and Norman M. White at McGill. I was fortunate to receive a full scholarship from a newspaper company in Japan and, later, another scholarship from the Government of Canada to complete my PhD at McGill. These first pivotal events set the stage for my career in science.

Figure 1. Noboru Hiroi, PhD, University of Texas Health Science Center at San Antonio, USA.

We would like to know more about your career trajectory leading up to your most relevant leadership role. What defining moments channeled you toward that leadership responsibility?

My PhD thesis work was focused on the anatomical loci critical for amphetamine's addictive properties. After completing my PhD thesis in 1991, I was offered postdoctoral positions in labs that were working on anatomical substrates of addictive substances at the University of British Columbia (Vancouver, British Columbia, Canada), Cambridge University (Cambridge, United Kingdom), and the Massachusetts Institute of Technology (MIT, Cambridge, MA). I chose the laboratory of Professor Ann M. Graybiel at MIT to expand my graduate training in behavioral neuroscience to the compartmentalization of the striatum, a field pioneered by Dr. Graybiel and others. At MIT, I was involved in a collaborative project with Dr. Susumu Tonegawa, one of the pioneers who introduced genetically engineered mice in studies of the role of genes in behavior. I completed a second postdoctoral program in the laboratory of Dr. Eric Nestler at Yale University (New Haven, Connecticut, USA) to further develop my skills in the molecular analyses of addictive behaviors in genetically engineered mouse models. My work there involved determining the roles of two genes, FBJ murine osteosarcoma viral oncogene homolog B (*FosB*) and dopamine- and cyclic adenosine monophosphate–regulated phosphoprotein, Mr 32 kDa (*DARPP-32*), in genetic knockout mouse models, in collaboration with Dr. Michael E. Greenberg at Harvard and Dr. Paul Greengard at Rockefeller University.

I was recruited to Albert Einstein College of Medicine in New York in 1998 as an independent junior faculty member to further strengthen its addiction research program. However, I was soon fascinated by my colleagues' work on patients who carried copy-number variants (CNVs) at human chromosome 22q11.2. Patients with these CNVs exhibit schizophrenia, intellectual disability, and autism spectrum disorder at rates far above what is expected in the general population. It became apparent that these patients represented genetically identifiable cases of mental disorders, which was too interesting a topic to pass on, and I started a new project exploring this association in 1999. My postdoctoral training in the integrative use of genetically engineered mice enabled me to contribute to the work of Einstein's 22q11 research team. I was fortunate to collaborate with many 22q11 pioneers, including Drs. Raju Kucherlapati, Bernice Morrow, and many others.

As my mouse work developed, I started expanding the level of analysis by forming a team of investigators specializing in the imaging of mouse brains, computational modeling, and cell models. This collaborative team grew to involve many investigators outside Einstein, including groups in Ireland and Japan. I currently organize an international team with those investigators.

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Published online: 16 February 2024.

What kind of impact do you hope to achieve in your field by focusing on your specific research topics?
I hope to increase the knowledge of cellular and molecular substrates for 22q11.2 CNV-linked psychiatric disorders so that the implementation of precision medicine in psychiatry can become a reality.

Could you tell us more about your current scholarly focal points within your chosen field of science?
My current point of focus is to elucidate and finely define the cellular and developmental origins of cognitive deficits commonly affected in CNV-associated cases of schizophrenia, autism, and intellectual disabilities. The ultimate validation of our findings in cell and mouse models would come when therapeutic options developed from the mechanistic understanding derived in these model systems prove to be effective for treating highly specific dimensions of mental disorders, which would be my dream. Even if potential therapeutic options turn out to be not effective, the negative outcomes would further motivate me to explore other potential cellular and developmental mechanisms of mental illness in model systems.

What habits and values did you develop during your academic studies or subsequent postdoctoral experiences that you uphold within your own research environment?
From my thesis mentor, Norman M. White, I learned to stick to my own ideas, even when they are not well accepted. I learned scientific rigor from Ann M. Graybiel and the importance of visions from my collaboration with Susumu Tonegawa at MIT. I learned cutting-edge molecular approaches from Eric Nestler at Yale. From all of my major scientific mentors, I learned the importance of continuously incorporating new techniques and ideas into my project.

At Genomic Press, we prioritize fostering research endeavors based solely on their inherent merit, uninfluenced by geography or the researchers' personal or demographic traits. Are there particular cultural facets within the scientific community that warrant transformative scrutiny, or is there a cause within science that deeply stirs your passions?
The current trend, which I do not particularly appreciate, is that articles consistent with prevailing dogmas tend to populate major high-impact journals and those inconsistent with these prevailing dogmas are not published in prominent journals. This trend is exacerbated by the geographic locations of the authors. Authors who do not reside in countries where dogmas are popular might be published less frequently, if the reviewers of their work are from countries where the dogmas are widely held. I am passionate about promoting work that does not support the prevailing concepts.

What do you most enjoy in your capacity as an academic and research leader?
The most enjoyable moments are the times when I discuss new ideas with my colleagues.

Outside professional confines, how do you prefer to allocate your leisure moments, or conversely, in what manner would you envision spending these moments given a choice?
My mother is housed in a care facility in Japan that specializes in Alzheimer's disease. Whenever I have a chance, I try to fly to see her.

Part 2: Noboru Hiroi – Selected questions from the Proust Questionnaire[1]

What is your idea of perfect happiness?
For me, perfect happiness involves seeing that kind of happiness in my family and two dogs.

What is your greatest fear?
My mother is currently afflicted with Alzheimer's disease. The prospect of developing the same condition at some point in my life is currently my greatest fear.

Which living person do you most admire?
There are too many to pick a few.

What is your greatest extravagance?
My greatest extravagance involves sharing superb Japanese food with my colleagues and collaborators.

What are you most proud of?
I am trying to achieve this state of mind about my work by the time I die or cease to function intellectually.

What is your greatest regret?
I tend to forget what I regret.

What is the quality you most admire in people?
One of the most admirable qualities in people is their ability to achieve goals despite adversity. I gave my son the middle name Moses. You get the idea of what qualities I admire in people.

What do you consider the most overrated virtue?
I am not sure. It depends on certain perspectives, and they differ individually.

What is your favorite occupation?
My current occupation is my favorite. This job is what I dreamed of as a child.

Where would you most like to live?
I would live anywhere I can collaborate with good people until I develop dementia or retire. After retirement or developing dementia, I would most likely live in Japan to enjoy the great food and hot springs. Moreover, Japan offers good, affordable medical care and care facilities. Major surgeries and good care facilities in the United States would cost me a fortune.

What is your most treasured possession?
I do not treasure physical possessions. Seeing my father's belongings after he recently passed away made me realize that physical possessions do not mean much. I cannot "possess" nonphysical things. My family is not my "possession." Therefore, I cannot think of any treasured possessions.

When and where were you happiest? And why were so happy then?
I am happiest now. I have never been this happy. Relatively speaking, my life was not so great earlier.

What is your most marked characteristic?
I tend to speak honestly and frankly, even to the extent that my speech may be blunt and abrasive at times.

Among your talents, which one gives you a competitive edge?
I tend to say what I think is true, even if it makes others uncomfortable or infuriated. That is the only thing I can think of, if you count it as a talent.

[1] In the late nineteenth century various questionnaires were a popular diversion designed to discover new things about old friends. What is now known as the 35-question Proust Questionnaire became famous after Marcel Proust's answers to these questions were found and published posthumously. Proust answered the questions twice, at ages 14 and 20. Multiple other historical and contemporary figures have answered the Proust Questionnaire, such as Oscar Wilde, Karl Marx, Arthur Conan Doyle, Stéphane Mallarmé, Paul Cézanne, Martin Boucher, Hugh Jackman, David Bowie, and Zendaya. The Proust Questionnaire is often used to interview celebrities: the idea is that by answering these questions an individual will reveal his or her true nature. We have condensed the Proust Questionnaire by reducing the number of questions and slightly rewording some. These curated questions aim to provide insights into the individual's inner world, ranging from notions of happiness and fear to aspirations and inspirations.

What is a personality/characteristic trait you wish you had?
I do not wish to have something I do not have or am incapable of having.

What do you consider your greatest achievement?
My greatest achievement so far is that I have survived as a researcher.

What do you most value in your friends?
The attributes I most value in my friends are that they are forgiving and not judgmental.

Who are your favorite writers?
I enjoy many nonfiction writers.

Who are your heroes of fiction?
I don't like heroes in fiction or fictional worlds. I do not like Disney or any other theme parks that include heroes. If anything, I prefer villains. They are tough.

Who are your heroes in real life?
Heroes in real life are those who sacrifice everything for the benefit and well-being of others.

What aphorism or motto best encapsulates your life philosophy?
The essence of life described in Ecclesiastes best encapsulates my life philosophy. Despite its seemingly pessimistic view of life, its conclusion is that one should nevertheless pursue wisdom because that is what providence dictates.

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VIEWPOINT

Why stating hypotheses in grant writing is usually necessary

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Keywords: Hypothesis evaluation, research methodology, philosophical perspectives in science, scientific discourse, knowledge evolution

In this viewpoint, we explore the provocative argument by Hernán and Greenland, presented in JAMA, regarding the traditional necessity of stating hypotheses in grant applications. They propose that this convention may hinder the explorative nature of research, calling for a reevaluation that could impact global research practices and methodologies. Hypotheses provide a structured framework crucial for clarifying research questions and facilitating successful funding. However, Hernán and Greenland merge grant writing with research execution, potentially undervaluing the strategic role of hypotheses. We discuss the perspectives of philosophers Karl Popper and Thomas Kuhn, emphasizing the essential role of hypotheses in fostering scientific progress through critical scrutiny and paradigm shifts. While acknowledging the value in Hernán and Greenland's flexibility for data-driven research, we assert that hypotheses remain fundamental in guiding scientific inquiry, balancing innovation with traditional rigor. Our discussion aims to contribute to the evolution of research methodologies, ensuring they are both innovative and grounded in systematic, hypothesis-driven approaches.

In their thought-provoking commentary published in *JAMA* (1), Miguel A. Hernán and Sander Greenland propose a reevaluation of the traditional necessity to state hypotheses in grant applications, suggesting that this practice might be unnecessary and even detrimental to the essence of research to explore effects with precision and openness. Our motivation to engage with Hernán and Greenland's discourse, particularly given its publication in a prestigious platform like *JAMA*, stems from an understanding of the profound impact this debate can have on clinical practices and research methodologies. The conversation extends beyond academic discourse, affecting how research is conceptualized, funded, and executed globally. Engaging in this dialogue is essential for developing research methodologies that combine innovation with the rigor necessary for significant advancements in medical science and beyond.

The original purpose of the Hernán and Greenland article, as inferred from its title, appears to focus on the role of hypotheses in grant writing. However, the content extends beyond this to encompass the implementation of research, blurring the lines between these distinct phases. In grant writing, hypotheses are crucial as they encapsulate the research question, direction, and rationale, providing a clear and structured framework for the study (2–4). They serve as foundational elements that guide the research's conceptual and analytical trajectory, facilitating successful grant acquisition.

However, Hernán and Greenland's blend of the grant-writing process with research execution overlooks the foundational role hypotheses play in the former. While their call for flexibility and data-driven approaches in research execution is valid and valuable, it somewhat diminishes the importance of a well-articulated hypothesis in securing grant funding. This overlook can lead to underestimation of the strategic importance of hypotheses in guiding the research journey, accommodating new data, and fostering unanticipated discoveries.

Expanding upon the philosophical perspectives of Karl Popper and Thomas Kuhn provides a richer understanding of this debate. Karl Popper and Thomas Kuhn are two of the 20th century's most influential philosophers of science. Each offers distinct perspectives on the role of hypotheses in scientific progress and the dynamics of paradigm shifts. Popper, known for his theory of falsifiability (5), argues that scientific theories should be framed in such a way that they can be rigorously tested and potentially disproven. According to Popper, the growth of scientific knowledge is an evolutionary process driven by the cycle of conjectures and refutations. He proposes that scientists put forward bold hypotheses and then attempt to falsify them. In this view, hypotheses are crucial as they offer clear, testable propositions that challenge the status quo. Popper contends that the inability to falsify a hypothesis does not confirm it as accurate but merely upholds it as the best approximation of truth currently available. Thus, for Popper, the hypothesis-driven approach is central to scientific discovery, as it encourages robust testing and critical scrutiny, leading to the elimination of errors and the advancement of knowledge.

On the other hand, Kuhn introduces the concept of scientific paradigms (6, 7) which he defines as universally recognized scientific achievements that, for a time, provide model problems and solutions to a community of practitioners. According to Kuhn, normal science operates within the confines of the current paradigm, focusing on solving puzzles that the paradigm delineates. However, when the paradigm encounters anomalies, it cannot be explained, this may lead to a scientific crisis and the eventual emergence of a new paradigm—a paradigm shift. For Kuhn, hypotheses are embedded within the prevailing scientific paradigms, guiding what questions scientists ask and how they interpret data. He suggests that significant scientific progress—paradigm shifts—occurs not just by accumulating facts or disproving hypotheses within the current paradigm, but by fundamentally changing the conceptual framework through which scientists view the world.

Thus, from both Popper's and Kuhn's perspectives, hypothesis-driven approaches are fundamental to the dynamics of scientific progress. They support the systematic and critical examination of our theories and practices, promoting continuous improvement and adaptation in our quest to understand the universe. These approaches encourage not only the refinement of existing knowledge within current paradigms but also the revolutionary shifts that redefine scientific understanding. In essence, by fostering a rigorous, question-driven approach to research, hypotheses play a vital role in both the evolutionary and revolutionary aspects of scientific advancement.

In contemporary scientific research, a clear distinction emerges between traditional hypothesis-driven studies and hypothesis-free investigations typical of 'big data' approaches, such as genome-wide association studies (GWAS) (8). Traditional methods, deeply rooted in specific, testable hypotheses, remain essential for targeted scientific inquiries. Conversely, GWAS and similar big data methodologies analyze extensive datasets to identify potential correlations without initial hypotheses. These explorations, while not immediately grounded in hypothesis testing, often generate findings that necessitate subsequent hypothesis-driven research. Such sequential approaches ensure that statistically significant results from large-scale data analysis are rigorously tested for

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their biological significance, thereby bridging the gap between statistical discovery and biomedical insight. This iterative cycle of discovery and validation embodies the dynamism and adaptability of modern scientific practice.

While Hernán and Greenland raise significant points that warrant serious consideration, it is essential to reflect on the broader implications of their arguments, particularly in the context of their publication in a high-impact journal like *JAMA*. The discourse surrounding the role of hypotheses in scientific research is vital, as it shapes the future of how we approach, understand, and solve the complex problems facing the medical and scientific communities. It is our hope that by adding our voice to this conversation, we can contribute to the ongoing evolution of research methodologies that are both innovative and grounded in the robust traditions of scientific inquiry.

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BENCH TO BEDSIDE

The importance of elderly genomes

Mayana Zatz[1]

The difficulty in classifying a rare genetic variant as "likely pathogenic," "likely benign," or VUS (variant of unknown significance) represents a significant challenge in genetic counseling (GC) when trying to establish a diagnosis or as a result of incidental findings. This classification may impact the communication of prognosis in late-onset conditions, such as neuromuscular disorders, and the consultants' reproductive decisions regarding future offspring. Here, we report two unrelated families, one Brazilian and one of East Asian ancestry, where a rare and previously unreported deletion in the dystrophin gene was identified. In these two families, the analysis of older male relatives (from 56 to 89 years old) who were fully asymptomatic provided relevant information to their families about the potential pathogenicity of this dystrophin variant. These cases support our previous suggestion highlighting the relevance of genome sequencing of older healthy individuals or family members, above the age of 50 and going into the 80's ad 90's, and the importance of sharing new relevant information for decision-making with families who previously underwent genetic counseling. In addition, these case reports contribute to the classification of VUS, enhancing our knowledge of the impact of specific mutations in functional studies.

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Keywords: Becker muscular dystrophy, Duchenne muscular dystrophy, exome sequencing, genetic counselling, genomics, whole genome sequencing

Next-generation sequencing (NGS) has allowed immense improvement in diagnosing genetic disorders, facilitating precision medicine. Furthermore, the current cost of whole exome sequencing (WES) and whole genome sequencing (WGS) makes them increasingly accessible diagnostic tools. However, we frequently have to deal with variants of unknown significance (VUS), which could cause a major illness or just be a rare genetic variant not yet deposited in the international genomic data banks.

Another ethical challenge geneticists face when sequencing a genome is accidental findings that could be utterly unrelated to the disease of the problem. For example, a mutation in the *BRCA1* gene, responsible for breast cancer, in an 8-year-old boy with an undiagnosed myopathy. Should the proband or the family be informed? The American College of Medical Genetics and Genomics (ACMG) published a list of genes and genetic variants that should be reported as incidental findings (or secondary findings) when they are discovered during genomic testing, even if they are unrelated to the suspected diagnosis (1, 2).

Our strategy has been to sequence the genome of healthy elderly individuals in Brazil, as those sequences could (1) contribute to databanks of our admixed Brazilian population, (2) help to classify the pathogenicity of rare unknown variants, and (3) provide essential insights on conditions that are prevalent later in life, such as hypertension, type 2 diabetes, Parkinson's, and cancer, among others. To pursue this strategy, in 2008, we launched the 80+ project, aiming to sequence the genomes of older Brazilians. A first draft, with 609 exomes, was published in 2017 (3), and a second study, including WGS of 1171 individuals, was published in 2022 (4), representing the most extensive genomic databank of older individuals in Latin America.

A comment in *Cell* published several years after our first study was initiated called attention to the importance of studying the genomes of admixed populations, as available databanks have been constructed mainly with individuals of European ancestry (5). Indeed, in our recent WGS study of more than 1000 individuals, we identified 2 million genetic variants not reported previously. More recently, the All of Us Research Program (6), a longitudinal cohort study aiming to enroll a diverse group of at least one million individuals across the United States, involved 77% of participants from communities that are historically under-represented in biomedical research and 46% individuals from under-represented racial and ethnic minorities. The All of Us Research Program identified more than 1 billion genetic variants, including more than 275 million previously unreported ones. This reinforces the value of studying the genomes of admixed populations. In addition to population genetic data banks, the genome study of older probands' relatives can be extremely valuable in real-life decision-making, as illustrated by two examples below.

Case 1

In 2012, a 44-year-old man was referred to our center because he had a mutation in the dystrophin gene, which was identified in a genetic center in the United States. He was perfectly healthy and robust, but he was investigated as a result of that accidental finding because his 10-year-old daughter had a diagnosis of coloboma and some hearing difficulties (7). The genome study of the young girl did not uncover any variant that could explain her condition. However, it revealed that she carried an unrelated mutation in the dystrophin gene, encompassing exons 38–44, inherited from her 44-year-old father. Most mutations in the dystrophin gene are responsible for Duchenne muscular dystrophy (DMD), a severe lethal condition that affects about 1 in 5000 male newborns (8). Those are disruptive mutations that result in the absence of muscle dystrophin. Affected boys usually lose ambulation by age 10–12 and are entirely dependent on all activities in their second decade. However, some mutations can result in a partially functional dystrophin and a milder but highly variable phenotype, as seen in Becker muscular dystrophy (BMD). Depending on the type and site of the mutation along the gene, BMD patients can be confined to a wheelchair around age 16 or remain ambulant in their sixties or seventies. For example, it is known that some mutations in the rod domain (central part of the gene) that maintain the RNA reading frame (in-frame deletions) can cause only cardiopathy later in life but no muscular weakness. Therefore, mutations in the dystrophin gene should be classified as dystrophinopathies and not Duchenne mutations, as they are responsible for a wide range of clinical variability.

The problem in this case is that the dystrophin mutation found in our proband had never been reported before. Could it be responsible for a late-onset disorder, or was it just a likely benign rare variant? Although he was healthy and strong at age 44, he wanted to know whether he might develop muscular weakness later in life. If he had carried a novel

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mutation, it would not have been possible to anticipate his clinical status later in life. The only alternative was to investigate his older relatives, hoping their genomic data might be informative. In other words, we needed to investigate whether those elderly relatives carried the same dystrophin mutation. That was the case: we studied several family members and found out that the proband's mother and one maternal uncle, who was 56 years old at that time, also carried the same mutation, and they were asymptomatic. It was good news. We published this case report with a take-home message: if you want to sequence your genome, keep your older relatives' DNA. They can bring important information (7).

Case 2

More recently, I received an email from a young woman of East Asian ancestry who wrote to me because she discovered that she carries a DMD mutation encompassing exons 38–44, the same rare deletion of the Brazilian family in case 1. Her mutation had been inherited from her 60-year-old mother. Because of the lack of information in genome data banks, the mutation was classified as likely to be pathogenic, and she underwent an abortion at 27 weeks of pregnancy. She wrote in her email that this "led her to be devastated, but also to conduct tons of research." Searching references, she discovered that her mutation was the same in our previously reported family, and she wanted more information about our case. Her questions were: (1) could you please provide more context on what "asymptomatic" means for that family? (2) Do they not show any signs of DMD/BMD, including no signs of CK increase/cardiomyopathy? (3) If this research shows exon deletion 38–44 is asymptomatic for this Brazilian family, can I safely assume it will also be asymptomatic for my family? (4) What is the current status of your patients 12 years after your report? (5) How much should I (she) be worried about this mutation in my (her) future offspring?

Our report reinforcing the importance of testing older relatives prompted her to study her grandparents. Her maternal grandmother was already deceased, and she had three brothers who refused to be tested. However, her maternal grandfather underwent genetic testing, which revealed that he carries the 38–44 mutation. It could not be better news since he is currently 89 years old, fully ambulant, and has no cardiomyopathy.

Asymptomatic genetic variants in patients of different ethnic backgrounds: "VUS or likely benign?

Following these last genetic results, I contacted the Brazilian family to share the excellent news about the healthy 89-year-old man carrying the 38–44 deletion, and they informed me that they also continue to be healthy and strong. Our proband and his maternal uncle carrying the dystrophin deletion are currently 56 and 68, respectively. The new observation that this same variant is not associated with any muscular weakness in two families with different ethnic backgrounds supports the hypothesis that it is a "likely benign" variant. However, some geneticists would still classify it as a VUS. Most importantly, it reinforces the relevance of genomic screening of older populations and probands' family members.

The pathogenicity of VUSs can also be studied using in *silico* strategies that include computational structural biology or in *vivo* experiments in which a new variant is created via CRISPR and inserted in a living organism. However, we believe that such models will lack the input from other putative protective variants; moreover, the outcomes of gene-gene interactions may be missed. Therefore, we advocate for the study of elderly genomes as a key tool to determine the clinical significance of VUSs.

In a recent review of the literature and database, Fortunate et al. reported 22 cases of patients who carried *in-frame* deletions in the dystrophin gene and were fully asymptomatic (9). They were older than 43, while the three individuals reported were older than 55. According to Fortunate et al., some deletions should be carefully considered when identified as incidental findings, and genetic counseling must always be offered to help interpret these rare dystrophin genotypes. Indeed, in the current case, sharing new data with our family was very helpful in their decision about future offspring, which also reinforces the importance of re-visiting previously counseled families with new, relevant information.

Fowler and Reham recently questioned whether VUS would still be present by 2030 (10). They suggest that investing in eliminating VUSs is worthwhile because their predominance remains one of the biggest challenges to precision genomic medicine. Sharing case reports such as those synthesized here can not only bring relief to families with such genetic variants but can also contribute to classifying the pathogenicity of VUS and rare variants.

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THOUGHT LEADERS INVITED REVIEW

The genetics of cognition in schizophrenia

Michael J. Owen[1], and Michael C. O'Donovan[1]

This conceptual review focuses on recent insights into the nature of the relationship between genetic predisposition and cognitive impairment as risk factors for schizophrenia, and the factors that influence the degree of cognitive impairment in those with the disorder. There is clear evidence that premorbid cognitive impairment is frequently present in those who develop schizophrenia, and, across the range of abilities, poorer premorbid cognition is associated with higher liability to the disorder. Evidence from genetic and population studies strongly supports the hypothesis that premorbid cognitive impairment is a marker for underlying neurodevelopmental risk factors for the disorder, rather than a prodromal manifestation. The premorbid cognitive deficit seems to be largely explained by non-familial factors rather than by familial factors that jointly influence liability to schizophrenia and cognitive ability, and these non-familial risk factors appear act to sensitize individuals to familial risk. There is also evidence that neurodevelopmental risk may be better indexed by the degree to which premorbid cognitive ability deviates from familial expectations than by cognitive ability *per se*. Premorbid cognitive impairment thus does not itself lie on the causal pathway to schizophrenia, rather it is a marker of a neurodevelopmental abnormality that is substantially non-familial, and which increases risk for schizophrenia. Genetic risk factors, including both common and rare alleles, that influence IQ in the general population also contribute both to liability for schizophrenia and to the degree of cognitive impairment in those with the disorder. There is also evidence for further decline in cognitive function after diagnosis in some individuals as well as an increased risk of dementia. This does not appear to reflect substantial shared heritability with neurodegenerative disorders, but the causes of postonset cognitive decline and its relationship to schizophrenia pathophysiology remain uncertain.

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Introduction: Cognitive Impairment in Schizophrenia

Schizophrenia is diagnosed based on the presence of core positive (psychotic), negative and disorganized symptoms (1). Individuals meeting diagnostic criteria show considerable heterogeneity in these and other clinical features, as well as in course and outcome (2). Comparisons between people with schizophrenia and controls reveal groupwise impairments in most aspects of cognitive function (3, 4) including IQ, which is on average reduced by approximately 1 SD in cases (5). Again, there is enormous variation between individuals in the extent of the impairment present, and a diagnosis of schizophrenia does not preclude estimates of cognitive ability that are above, and sometimes markedly above, average (6, 7). Cognitive impairment is not a core symptom of schizophrenia in DSM5 or ICD11, but as it is strongly associated with poorer functional outcomes in areas such as work, independent living, and social integration (3), it is of great importance to those with the disorder, and to those who are involved in their care and management, A recent review has broadly considered evidence concerning the etiology, pathogenesis, and treatment of cognitive impairment in schizophrenia (4). The current conceptual review is more circumscribed and focuses on additional important insights provided by recent studies into the nature of the relationship between genetic risk and cognitive impairment as risk factors for schizophrenia, and the factors that influence the degree of cognitive impairment in those with the disorder.

Many of the studies we review have been based upon derived measures of general cognitive function such as IQ rather than upon performance on specific tests measuring specific domains of cognitive function. Given that impairments in most aspects of cognitive function are associated with schizophrenia (3), we believe that such global measures are informative for the specific questions we aim to address. Accordingly, we use the term cognitive impairment interchangeably with low IQ rather than to indicate impairment in specific cognitive domains.

People who receive a diagnosis of schizophrenia frequently exhibit "premorbid" cognitive impairments before psychosis is manifest (8), including an average IQ that is approximately 0.5 SD below that of controls (5). There is also evidence that the severity of premorbid impairment is associated with earlier onset of schizophrenia (9). Impairments are evident in childhood (5, 10–12) and appear to represent a failure of typical developmental acquisition of function rather than a deterioration *per se* as might be expected of a degenerative process (12). While the evidence suggests that the developmental trajectories of those who develop schizophrenia diverge from controls many years before psychosis emerges, it remains unclear whether this is preceded by a period of normal development and, if so, exactly when divergence begins (10, 12). Alongside other premorbid developmental and environmental risk factors, the findings point to schizophrenia having origins in disturbances of neurodevelopment, one manifestation of which is impaired cognition prior to onset of psychosis (13–15).

While the weight of evidence supports a neurodevelopmental explanation for premorbid cognitive impairment in schizophrenia, the nature of the relationship between the two is uncertain. In principle, there are three possibilities. First, premorbid cognitive impairment could be a prodromal manifestation of an insidious onset of schizophrenia. Secondly, it could be a causal risk factor mediating the effects of genetic or environmental risk on the development of schizophrenia, a so-called intermediate phenotype or endophenotype. Thirdly, it might be a risk indicator that results from the pleiotropic effects of an underlying neurodevelopmental abnormality that independently increases risk of the subsequent emergence of schizophrenia. In this latter instance, premorbid cognitive impairment would be a marker of the presence or extent of a neurodevelopmental abnormality but not itself lie on the causal pathway to schizophrenia.

Some studies comparing cognitive function in the same individuals before and after onset of schizophrenia (5, 16–18) suggest that, as well

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as premorbid deficits, there is further decline in function after diagnosis in some individuals. However, this remains a controversial area and two meta-analyses of longitudinal studies found no evidence for postonset decline in the first 5 years after onset (19, 20). Moreover, a recent umbrella review concluded that most of the reviews assessed point to no decline of cognitive function over short to medium time frames (21). Nevertheless, studies over longer follow-up periods have found evidence for accelerated cognitive decline (18) and substantially increased risks of dementia have been reported in those with schizophrenia (22, 23). Some have argued that this later decline points to the operation of a primary neurodegenerative process in schizophrenia (24), whereas others have argued that many people with schizophrenia do not progressively deteriorate and pointed to the possibility that, where decline does occur, it reflects non-specific factors secondary to schizophrenia such as antipsychotic exposure, metabolic syndrome, smoking and other substance abuse and various social confounders (25, 26).

Finally, the considerable variation in cognitive impairment seen in people with schizophrenia raises the question as to what extent this might be associated with the same genetic factors that influence variation in cognitive ability in the general population, or whether factors that are relatively specific to schizophrenia operate.

Genetic Architectures of Schizophrenia and Intelligence

Schizophrenia is highly heritable and polygenic, with risk conferred by alleles across the frequency spectrum (27). Genome-wide association studies have so far identified 287 loci that contain common risk alleles of small effect (28), but many more exist, likely thousands, that are collectively responsible for around a third of genetic liability (29, 30). In addition, at least eight rare copy-number variants (CNVs) have been identified that confer substantial individual risk (31, 32). More recently, exome sequencing studies have shown rare protein truncating variants and damaging missense mutations (33–39) can confer large effects on risk of schizophrenia. The largest exome wide sequencing study of the disorder to date reported by the Schizophrenia Exome Meta-Analysis Consortium (SCHEMA) (38) identified 10 genes with an exome-wide significant excess burden of these classes of mutation in cases. An additional two genes met this significance threshold in a focused sequencing study targeting genes that had shown some evidence for association in a preliminary analysis of the SCHEMA exome wide study (40).

Genomic studies of common variants have found evidence for genetic correlation between schizophrenia and other psychiatric conditions and personality traits, with the strongest relationship seen with bipolar disorder where the shared heritability is approximately 0.7 (41). In contrast, for rare variants, while there is evidence for overlaps with bipolar disorder (42), so far, the genetic overlaps are more prominent between schizophrenia and childhood onset neurodevelopmental disorders (NDDs) which, like schizophrenia, are also associated with cognitive impairment: all known schizophrenia associated CNVs have been implicated in intellectual disability, and some have also been implicated in autism (43). People with schizophrenia are also enriched for ultra-rare damaging mutations in NDD-associated genes (33, 36–39), including specific mutations that are pathogenic for NDDs (44). Finally, common schizophrenia risk alleles are enriched in genes implicated by rare variant studies of NDDs (28).

Intelligence is moderately heritable in the general population (45). Genomic studies have shown that, like schizophrenia and as predicted (45), it is highly polygenic, and impacted by alleles across the frequency spectrum including many common variants of small effect (46), as well as rare alleles including chromosomal abnormalities, CNVs, and rare coding variants, some of which are also associated with schizophrenia and other NDDs (47–50).

The Relationship Between Genetic Risk and Cognitive Impairment as Risk Factors for Schizophrenia

Family and Population Studies

There is evidence from many individual studies, supported by meta-analyses, that unaffected first-degree relatives (FDRs) of those with schizophrenia show generalized impairments of cognitive function but to a lesser degree than those seen in probands (51, 52). However, such findings are not universal. One study of schizophrenia cases and their relatives from relatively highly functioning families found that the siblings of probands did not differ in cognitive performance from a community control sample (53). In addition, a very large study of the Swedish population found no evidence for adolescent cognitive impairment in the siblings of people with schizophrenia (54). These findings raise the possibility that the role of familial risk factors for low IQ in schizophrenia may have been influenced by ascertainment bias in studies of probands and their relatives. Another possible confounder is that families ascertained for having a schizophrenia proband may have higher rates of exposure to environments with an impact on cognitive ability, for example cannabis abuse (55). Finally, there is evidence for assortative mating such that people with schizophrenia who have children on average have partners of lower cognitive ability than controls (56). As a result, assortative mating could contribute to, or even account for, deficits in FDRs rather than these reflecting a substantial overlap between the genetic risk for schizophrenia and low IQ. Thus, while many studies have found evidence for impaired cognition in FDRs, questions remain about how these findings should be interpreted. Genomic studies of parent-proband trios might throw light on this issue.

Population studies have further illuminated the causal relationship between premorbid IQ and genetic risk. In a large study of the Swedish population (57), risk of schizophrenia increased by 3.8% with each one-point decrease in premorbid IQ. The effects were stronger in the lower IQ range, but importantly, the association was monotonic, meaning at no point in the distribution was higher IQ associated with higher risk, although such an effect in people with exceptionally high IQ could not be excluded due to the rarity of those individuals. Overall, these findings, and the magnitude of the effect, were almost identical to those obtained in a meta-analysis of earlier population studies (9). The Swedish study (57) noted that the association between premorbid IQ and risk of schizophrenia was of similar magnitude when onsets within 5 years of testing were excluded, suggesting that it does not reflect potential prodromal effects of declining IQ associated with insidious onset. Again, these findings are in accord with those from earlier population studies which excluded onsets immediately prior to testing (51). Finally, the Swedish study found that that risk of schizophrenia in people with high familial liability to the disorder was substantially modified by premorbid IQ, familial susceptibility having a much stronger impact on risk of illness in those with low IQ.

Since intelligence and schizophrenia are both familial and substantially heritable, the authors of the Swedish study (57) tested the hypothesis that the premorbid IQ-schizophrenia association might be the result of genetic (and family environment) factors that predispose to both traits. They undertook co-relative analyses, comparing the strength of the association between intelligence and schizophrenia within various classes of pairs of relatives whose IQs were different, that is not in the same decile. Siblings share more familial factors (genetic and environmental) than do more distal pairs of relatives, and therefore more of the difference in intelligence between them is likely to be attributable to non-familial factors than is the case for difference in intelligence between more distally related pairs. Accordingly, if the association between low IQ and schizophrenia is substantially due to genes or familial environments that influence both traits, the strength of this association within siblings will be less than in the more distantly related relative pairs. However, the findings were inconsistent with this; association between premorbid IQ and schizophrenia was as strong within siblings as within more distant relatives, and even between people in the general population who are effectively unrelated, suggesting the link between the traits is not the result of heritable genetic or familial environmental risk factors that jointly influence both traits. These findings have been supported by two recent population-based sibling studies (58, 59).

Evidence that non-familial factors may have an important influence on premorbid IQ deficits in schizophrenia also comes from studies showing that risk of schizophrenia may be better indexed by a measure of the extent to which an individual's premorbid cognitive performance is lower than expected based on estimates of familial cognitive aptitude than by their absolute premorbid cognitive performance *per se* (54, 60, 61). It is conceivable that such deviations from familial expectation might occur

in individuals who, by an unlucky roll of the genetic dice, inherit an excess of poorer cognition alleles that have pleiotropic effects on liability to schizophrenia. However, this interpretation was not supported by the finding that, when schizophrenia and control probands were matched for cognitive performance, the siblings of the schizophrenia probands had scholastic aptitudes and IQs that did not differ from population means and which were significantly higher than those of the siblings of control probands (54). Instead, this series of studies from Sweden (54, 57, 60) suggest that an important contribution to risk for schizophrenia comes from neurodevelopmental perturbations that impact cognitive development in people who develop schizophrenia that are not caused by familial factors that typically influence cognition within families. This general conclusion also is indirectly supported by evidence that the heritabilities of a range of cognitive abilities in schizophrenia seem to be lower than in the general population (61).

Overlap in Common Risk Alleles for Schizophrenia and IQ

There is evidence that some of the common alleles that influence cognitive ability in the general adult population also influence liability to schizophrenia, but the genetic correlation of −0.21 between the two is modest (46). A similar genetic correlation of −0.22 has also been reported between liability to schizophrenia and intelligence estimated in 16 year olds, an age prior to the typical onset of schizophrenia (62). These correlations suggest that, on average, common variant genetic effects associated with lower intelligence in the general population explain around 5% of liability to schizophrenia, an estimate consistent with a longitudinal population-based twin study which estimated this figure at 7% (63). These findings, based on very different study designs, converge on the conclusion that, while there is some shared genetic liability between IQ and risk of schizophrenia, the overlap is small, and in line with the conclusions from the Swedish family studies reviewed above. It should be noted that genetic correlations of this magnitude could potentially be the result of the type of cross-trait assortative mating (64) discussed above (56), as well as other sources of confounding (65), possibilities that warrant further study. Moreover, shared liability does not imply that risk of schizophrenia is mediated by the effects on cognitive ability, it may instead indicate the existence of pleiotropy whereby common alleles independently influence cognitive ability and liability to schizophrenia, perhaps by contributing to the pleiotropic neurodevelopmental perturbations discussed in the previous section.

A study (66) based on modeling the relationships between schizophrenia polygenic liability [indexed by polygenic risk score (PRS)], schizophrenia and cognition (expressed as latent traits) within families found that the best model solutions suggested around a third of genetic risk of schizophrenia could be explained through causal effects on cognition. However, the modeling does not appear to have accounted for the possibility of pleiotropy, and, as the authors noted, the limitations and assumptions mean that their approach cannot prove causality, and longitudinal designs and/or Mendelian randomization approaches are needed. However, to date, the required longitudinal studies have not been conducted and Mendelian randomization methods have not been able to clearly distinguish between causal and pleiotropic hypotheses (67). Moreover, a recent report that almost all variant sites that influence IQ in the general population also influence liability to schizophrenia, but that the specific alleles that increase schizophrenia risk are a mix of lower and higher IQ alleles, suggests that shared liability cannot indicate a simple causal link between lower IQ *per se*, and schizophrenia (68).

Genomic Studies of Variation in Cognitive Function in Schizophrenia

Common Alleles

Separate to the question about the relationship between alleles that influence cognition in the general population and those that influence liability to schizophrenia is what is the relationship of those sets of alleles to cognitive ability in people with the disorder?

There is strong evidence that alleles influencing IQ in the general population also have effects on cognitive ability in individuals with schizophrenia (69–71). In contrast, the evidence for a relationship between cognitive function in schizophrenia and common variant liability for the disorder is inconsistent (69, 70, 72–75). This inconsistency may reflect the relatively modest sample sizes and power of some of the studies as well as differences in duration of illness at the time of cognitive testing and the nature of the cognitive tests employed. Nevertheless, the results suggest that the effects of common schizophrenia risk alleles, as indexed by a polygenic risk score (PRS), on cognition in people with schizophrenia are at best small, and where direct comparisons have been made (69–71), considerably smaller than the effects of common alleles that influence IQ in the general population (as indexed by an IQ PRS). The IQ PRS explains around 9% of variance in IQ (76) in people with schizophrenia. This is similar to estimates in the general population (62), although one study suggested the variance explained in cases is less than in UK Biobank controls, albeit using different cognitive measures in cases and controls (70). These findings, together with those indicating that the genetic correlation between liability to schizophrenia and IQ is modest, and the findings from the large population family studies reviewed above, suggest that variation in premorbid cognitive function in people who later develop schizophrenia is not substantially the result of common genetic risk factors for schizophrenia. There is also clear evidence from genomic studies that common alleles that influence variance in cognitive ability in the general population also do so in people with the disorder. However, in the absence of direct comparisons of cases and controls based on identical cognitive assessments, it is unclear to what extent those alleles can explain the average premorbid cognitive deficit seen in people who develop schizophrenia.

Rare Variants

People with schizophrenia who carry schizophrenia associated CNVs tend to have worse cognitive function than those do not, the average difference between the two groups in measures of cognitive performance being around 0.5–1.0 SD (77). The greatest reductions in cognitive ability are seen in those with CNVs spanning loss-of-function intolerant (LoFI) genes, that is genes in which loss-of-function mutations are highly disadvantageous for reproductive fitness. There is also evidence that in people with schizophrenia, rare coding risk variants (RCVs), particularly protein-truncating variants in LoFI genes, are associated with reduced cognitive function (71, 76), poorer educational attainment (33, 78) and an increased risk of comorbid intellectual disability (37, 78). The effects of rare variants on cognition are largely manifest before illness onset (see below), suggesting that their effects impact neurodevelopmental processes. Further support for neurodevelopmental effects comes from the observation that the effects of RCVs on cognitive function in schizophrenia are on average greater for those within genes that have been implicated in childhood NDDs than for RCVs in other mutation intolerant genes (37, 76, 78). Given evidence reviewed above that non-familial effects are important, it is also notable that the effect sizes of mutations occurring *de novo* (which by definition are non-familial) are larger than those of transmitted mutations (38, 76, 78).

Premorbid versus Postonset Effects

Few genomic studies have included measures of both premorbid and current cognition in people with schizophrenia, but a small number of recent studies that have done so have begun to throw some light on the links between genetic risk factors and the timing of cognitive impairment. Most of the impacts of CNVs, RCVs, and IQ PRS on cognition are premorbid (69, 71, 76, 77). In contrast, there is some evidence that the relatively small effects of common variant schizophrenia liability on cognition occur after onset (69) but do not influence premorbid cognitive ability (69, 71, 76). The findings that schizophrenia common variant liability, while not associated with premorbid impairment, may be associated with later impairment requires replication, but if confirmed, might either reflect effects on cognitive decline around the time of onset of psychosis or at some point thereafter. The findings from a longitudinal study that association between schizophrenia PRS and poorer cognition is stable across a 20-year follow-up period (75) would tend to suggest that the effects on cognition are most likely to occur around the time of onset.

Regarding the substantial increases in risk of dementia reported in schizophrenia mentioned above, it is notable that there is no evidence that schizophrenia shares heritability with Alzheimer's or Parkinson's

diseases, although there is weak evidence for some sharing with frontotemporal dementia (79–81).

Conclusions and Implications

The findings from genetic studies that we have described are relevant to three broad sets of issues. The first concerns the question of what accounts for the lower premorbid IQ seen in schizophrenia and the nature of the relationship between this impairment and other risk factors for schizophrenia. The second relates to what genetic factors influence the extent of cognitive impairment among those with schizophrenia. The third concerns the possible influence of genetic effects on cognitive decline in later life.

Premorbid Cognitive Impairment and Risk of Schizophrenia

Evidence reviewed above suggests that low premorbid IQ is a risk factor for schizophrenia and that this is not explained by prodromal effects of an insidious onset of the disorder. Moreover, rather than an "U"-shaped relationship between IQ and risk of the disorder, highest risk occurs in people with lowest IQ, lowest risk in those with the highest IQ, and on average, people who subsequently develop schizophrenia have a 0.5 SD deficit in premorbid IQ compared with the general population average.

In principle, the strong evidence that schizophrenia risk alleles can also influence cognitive ability in the general population provides a plausible explanation for low premorbid IQ. Intuitively attractive though this hypothesis may be, it is not supported by the evidence we have discussed. Common variant genetic liability to schizophrenia, which currently explains by far most of the attributable heritability of the disorder (82), does not appear to be associated with premorbid IQ (69, 71), a finding broadly consistent with the small genetic correlations observed between liability to psychosis and premorbid IQ in twins and between schizophrenia and IQ in the general population. Thus, the genomic evidence to date is consistent with that outlined above from genetic epidemiology in suggesting that the premorbid cognitive deficit in schizophrenia is largely explained by non-familial rather than familial factors that jointly influence liability to schizophrenia and cognitive ability. There is strong evidence these non-familial factors include *de novo* mutations (SNVs and CNVs), but these have been implicated in fewer than 5% of cases (82) and therefore other non-familial risk factors must contribute, including as yet unidentified *de novo* mutations, such as rare structural variants and non-coding variants, non-familial environmental factors, for example *in utero* or perinatal birth trauma and infections, and stochastic events (83) that contribute to variation in neurodevelopment.

Some caveats should be noted. First, it is conceivable that studies of the effects of schizophrenia PRS on cognition in cases may underestimate effects due to Berkson's paradox (84), also sometimes known as collider bias (84). Assuming PRS and low IQ to be at least partly independent risk factors, people with exposure to higher PRS will require less exposure to the risk conferred by low IQ in order to manifest the disorder, and vice versa. This can result in a spurious negative correlation between the two risk factors in case only studies, or, where a true population association exists, a reduction in the estimated effect size. Secondly, only a minority of schizophrenia heritability is currently attributable to known types of variant (82) and, in principle, classes of variants responsible for the unexplained heritability could show stronger associations with cognition, although the findings from studies of siblings and other relatives reviewed above (57–59) suggest that this is unlikely to be the case. Thirdly, the findings of the key genetic epidemiological studies require further replication.

Having established that low premorbid IQ is a risk factor for schizophrenia the next question is whether low IQ is *per se* causal or is instead a risk indicator of a pleiotropic neurodevelopmental abnormality that can (largely) independently manifest as low IQ in childhood and the emergence of schizophrenia in later life. The latter interpretation is supported by the observation that risk of schizophrenia is better indexed by the deviation of cognitive performance from that expected, than by absolute premorbid cognitive performance, which is contrary to the expectation if low IQ *per se* is directly causal. Molecular genetic studies of common and rare variation are also inconsistent with the idea that low IQ *per se* is on the causal pathway to schizophrenia. Thus, while all known schizophrenia-associated CNVs, and certain schizophrenia—associated RCVs are associated with low IQ, their impacts on risk in schizophrenia is not contingent on the presence of low IQ (41). The observation that common schizophrenia susceptibility alleles include those associated with higher IQ as well as lower IQ similarly suggests that there is no robust causal link between lower IQ and schizophrenia. There is, however, evidence that low premorbid IQ may sensitize individuals to familial risk factors (57) (Figure 1A) and there is a need to substantiate this finding and, if confirmed, explore possible mechanisms.

A further issue that warrants discussion is whether there is a neurodevelopmental subtype of schizophrenia characterized by premorbid cognitive impairment. We have discussed this elsewhere (44) and our view is that the evidence better supports the hypothesis that there is a spectrum of neurodevelopmental impairment in those with schizophrenia, rather than a clear distinction between a form of the disorder with cognitive impairment and one without (15). The monotonic change in risk of schizophrenia across the full IQ range, rather than there being an IQ threshold that is associated with a step change in liability, supports this (9, 57) as does the observation that people with lower cognitive ability in schizophrenia do not substantially differ from those with higher cognitive ability with respect to common alleles that confer risk to schizophrenia generally (70). Finally, in people with CNVs known to affect neurodevelopment, schizophrenia is the result of both the CNV and the common variant liability that is shared with general forms of the disorder (85, 86). While we do not believe that the data support the existence of a distinct neurodevelopmental subtype of schizophrenia, the extent of premorbid cognitive impairment, and more particularly the extent that this deviates from familial expectations, seems to index the degree of underlying neurodevelopmental impairment and may be an important clinical and prognostic marker within the disorder (Figure 1B).

We additionally note that while our focus here is schizophrenia, comparisons of the degree of premorbid deviation from familial cognitive aptitude (60) in different psychiatric disorders support the view that the neurodevelopmental continuum extends across a number of conditions, as previously proposed (15, 87). Thus, the effect size of deviation from familial cognitive aptitude was greatest for ASD, followed by schizophrenia and other non-affective psychoses, and least in bipolar disorder (60) supporting the suggestion (15, 87) that there is a gradient of neurodevelopmental pathology across neurodevelopmental and psychiatric disorders. According to this view schizophrenia occupies an intermediate position between childhood neurodevelopmental conditions and bipolar disorder. This helps explain why cognitive impairment is associated with schizophrenia but to a much lesser extent bipolar disorder despite the high common variant genetic correlation between the two conditions.

It is now important to identify the risk factors for, and the nature of, the neurodevelopmental abnormality underlying premorbid cognitive impairments.

It will also be important to determine how and when neurodevelopmental impairment moderates the impact of familial genetic risk for schizophrenia. One hypothesis with potential implications for interventions is that the effects of divergence from familial cognitive expectations on psychopathology might be mediated by the evocation of disrupted family dynamics and/or impairment of individual's self-esteem (88) (Figure 1).

Genetic Factors Influencing the Degree of Cognitive Impairment in Schizophrenia

As we have seen, recent genomic studies suggest that all classes of allele that influence liability to schizophrenia or to variance in IQ contribute to variation in premorbid cognition and to cross-sectional degree of cognitive impairment in people with established schizophrenia. These types of variant currently explain around 10% of variance in premorbid IQ, of which, as noted above, 90% is explained by IQ PRS and the remainder by rare CNVs and rare damaging coding variants in constrained genes. They explain less variance in cognition, around 6%, in those with established schizophrenia (76), but after allowing for the effects of premorbid cognition, this drops to around 1.6%, equally split between IQ and schizophrenia PRS. Thus, most of the genetic contribution to variation in cognition in

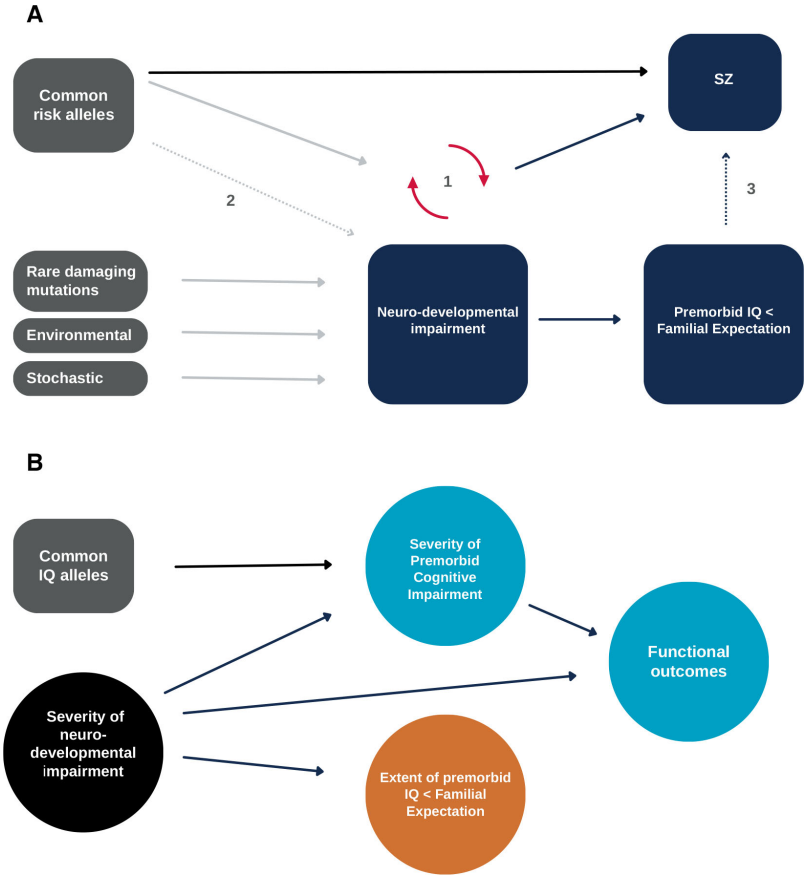

Figure 1. (A) A model of the relationship between premorbid cognitive impairment and risk of schizophrenia. The degree to which premorbid IQ deviates from familial expectations, rather than IQ *per se,* is a key risk indicator for schizophrenia. This deviation indexes an underlying neurodevelopmental impairment (NDI) that increases the risk of schizophrenia, and which sensitizes the individual to familial risk factors including common variant liability indexed by schizophrenia PRS as well as some other forms of transmitted genetic variant (1). The NDI reflects predominantly non-familial factors including environmental risk factors, stochastic factors and rare damaging de novo mutations. However, familial risk of schizophrenia likely contributes to NDI (2) given evidence that genes implicated by common risk variants overlap those associated with rare disruptive coding variants in schizophrenia, are enriched for genes implicated by such variants in NDDs (28) and evidence that they are enriched for genes with high expression specificity in developing fetal neuronal populations independently of those expressed in adulthood (89). An individual's deviation from familial cognitive aptitude expectation might impact on their psychopathology via influences on their self-esteem and family relationships (3). (B) Severity of premorbid cognitive impairment in schizophrenia. The severity of premorbid cognitive impairment in individuals with schizophrenia reflects both the contribution of common alleles that are associated with IQ in the general population indexed by IQ PRS and the severity of the underlying NDI which is indexed by the extent of premorbid cognitive impairment relative to familial expectations. The latter is likely to be an important marker of stratification within the disorder indicating a propensity to poor functional outcomes. The direct arrow from NDI to outcomes acknowledges that more research is needed to determine the extent to which different outcomes are mediated by cognitive impairment.

schizophrenia is mediated by effects that we have argued above are neurodevelopmental. Also note that, while rare mutations contribute only a small amount to variance in premorbid cognition, they are associated with relatively large impairments of cognitive function (76).

A caveat to our conclusion that most of the genetic influences on cognition in schizophrenia are premorbid and likely neurodevelopmental is that, given our incomplete understanding of genetic architecture, we cannot exclude the existence of as yet unknown types of risk variant that show a different balance of morbid and premorbid effects on cognition. The existence of such variants is plausible given evidence we have discussed that any effects on cognition of common risk alleles for schizophrenia seem to manifest postmorbidly rather than premorbidly (69). Another caveat is there may be specific genetic contributions to cognition in schizophrenia that are independent of those that confer liability to the disorder, or to intelligence in the general population.

Genetic Influences on Postonset Cognitive Decline

As we have seen, uncertainties remain concerning the extent to which there is cognitive decline after onset and if so what proportion of cases are affected and when the decline occurs. There is emerging evidence of decline in cognitive function after schizophrenia onset relative to age matched controls, which appears to be progressive over many years (18) and for substantially increased risks of dementia (22, 23). The presence of late life cognitive decline and increased risk of dementia in people with schizophrenia in the absence of increased genetic liability to dementia suggests that schizophrenia may lead to higher exposure, or greater vulnerability, to the same environmental risk factors that operate in the general population to increase risk of dementia, for example smoking, poor cardiovascular health, and lower cognitive reserve, the latter being a consequence of lower premorbid IQ, social isolation, and low rates of employment. A second explanation is that the increased risk of dementia reflects intrinsic pathogenic mechanisms related to schizophrenia, or environmental exposures, that are relatively specific to those with severe mental illness for example medication effects. This is an area that needs further research that includes measures of genetic and potential environmental risk factors and robust measures of cognition ideally over multiple timepoints (24, 26).

Limitations

An important limitation of many of the cited studies is that different measures of cognition have been used and analyses have typically been based upon derived measures of general cognitive function such as IQ rather than upon performance on specific tests measuring specific domains of cognitive function. In defense of this approach, the evidence suggests that reductions in most aspects of cognitive function are associated with schizophrenia (3) in line with an underlying neurodevelopmental impairment that impacts broadly on cognitive performance. However, it is possible that there are specific cognitive impairments that are important mediators of risk that will be identified by future research. Additionally, the measures of premorbid cognitive function used in many genomic studies have been indirect. Concern is mitigated to some extent by studies showing that such measures are strongly correlated with direct measures of premorbid IQ (90). However, we acknowledge that large longitudinal cohorts with direct measures of cognitive function would offer a better means of investigating how genetic and other risk factors influence cognitive function over the lifespan in those with schizophrenia. Finally, as we have noted, only around 10% of variance in cognition in schizophrenia is currently attributable to alleles of the classes so far studied, and even then, the vast majority of this is attributable to polygenic scores rather than specific causal alleles. Our inferences are therefore based on an incomplete understanding of the genetic architecture of cognition in the general population as well as in schizophrenia.

Conclusions

Despite these limitations and the caveats and uncertainties we have noted throughout, the available evidence from genetic studies has allowed us to identify some important conclusions and to propose a model of the relationship between premorbid cognitive impairment and risk of schizophrenia which best fits the current data (Figure 1). This model will no doubt need to be revised as further findings accumulate. In particular, it will be important to further replicate the finding that deviation from familial cognitive aptitude is a better risk indicator than IQ *per se* and to understand the genetic and environmental factors that underlie this deviation and how it interacts with genetic risk for schizophrenia. However, as it stands our model and the data upon which it is based have important implications for interpreting both endophenotype and animal model studies as well as for interventions aimed at improving cognitive function in schizophrenia.

The factors underlying cognitive decline after onset remain unclear and it is not apparent to what extent these are intrinsic to the disease process or secondary to schizophrenia such as antipsychotic exposure, metabolic syndrome, smoking and other substance abuse and various social confounders. Understanding how and when the effects on cognition, premorbid, and postonset, arise are key questions for research given the potential for prevention and early intervention.

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Author Contributions

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THOUGHT LEADERS INVITED REVIEW

Liver X and thyroid hormone receptors in neurodegeneration

Margaret Warner[1], Xiaoyu Song[1], and Jan-Åke Gustafsson[1,2]

The role of thyroid hormone (TH) in the development and function of the central nervous system (CNS) has been known for many years. However, the role of liver X receptors (LXRs) in TH function and protection against neuronal degeneration was not recognized until recently. The relationship between thyroid hormone receptors (TRs) and LXRs became apparent with the cloning of steroid hormone receptors, leading to the discovery of the nuclear receptor superfamily. This family includes not only receptors for classical steroid hormones but also many newly discovered ligand-activated nuclear receptors. LXRs and TRs regulate overlapping pathways in lipid and carbohydrate metabolism, as well as in overall CNS development and function. These CNS pathways include neuronal migration during cortical and cerebellar layering, myelination, oligodendrocyte maturation, microglial activation, and astrocyte functions. Furthermore, LXRs likely have unique functions, as evidenced by the inability of TH to compensate for microglial activation, oligodendrocyte maturation, spinal motor neuron death, and degeneration of retinal and cochlear neurons in LXRβ knockout mice. The common and unique functions of these two receptors are the subject of this review. We analyzed some of the most relevant literature on the regulation and function of LXRs and TRs and investigated why both receptors are required in the human body. We conclude that LXRs and TRs do not represent parallel pathways but rather constitute a single pathway through which the TH endocrine system regulates cholesterol homeostasis. Subsequently, LXRs, activated by cholesterol metabolites, function as a paracrine/autocrine system that modulates the target cell response to TH.

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Historical Perspective: LXRs

Liver X receptors (LXRs, LXRα, and LXRβ) belong to a subfamily of the nuclear receptor superfamily of ligand-activated transcription factors, which comprises 48 members in the human genome (1). Nuclear receptors play crucial roles in regulating metabolism, endocrine systems, and the development and function of the central nervous system (CNS). Although the functions of thyroid hormone (TH) have been studied for many years, LXRs were only discovered in the 1990s. Thyroid hormone receptors (TRs), TRα and TRβ, are differentially expressed in various tissues and have distinct roles in TH signaling (2). LXRβ (gene name *NR1H2*) was independently discovered by several laboratories (3–6) in 1996 and was initially designated as OR1, UR, NER, and RIP-15. It was later renamed LXRβ due to its homology with LXRα (also known as *NR1H3*), a receptor discovered in 1994 (7, 8).

LXRα has two major functions in the body: lipid metabolism in organs such as the liver, intestine, and adipose tissue, and regulation of the immune system, notably in macrophages (9). LXRβ has a broader tissue distribution than LXRα; while its expression in the liver is low, LXRβ is well expressed in immune system cells, glial cells in the CNS, colon, gallbladder, pancreatic islets, retina, and inner ear (10–16). Although it is expressed in very few neurons in the adult mouse brain (17), LXRβ is widely expressed in neurons of the fetal brain (18, 19). Both LXRα and LXRβ are expressed in the ovary, testis, prostate epithelium, and epididymis, where they play significant roles (20–23).

While the most well-studied function of LXRs is their role in cholesterol homeostasis (24), a function shared with TRs, cholesterol transport is just one of many transport functions of LXRs. Like TRs, LXRs regulate the transport of water by modulating aquaporins (25–29) and glucose through GLUT4 regulation (30–32). In addition, LXRs regulate the transport of THs and lactate through monocarboxylate transporters MCT8 and MCT10 (33). Transport of lactate into neurons is essential for neuronal nutrition, and its regulation by LXRβ (via MCT1) may explain the loss of neurons in LXR$\beta^{-/-}$ mice.

Classical hormones such as androgens, estrogens, progesterone, glucocorticoids, and thyroid hormone function in endocrine pathways where glands (such as the testis, ovaries, adrenal glands, and thyroid gland) secrete hormones into the bloodstream, which target organs receive via the vascular system. With the exception of the vitamin D receptor, more recently discovered members of the nuclear receptor superfamily are activated by ligands not secreted from endocrine glands but rather synthesized in various cells throughout the body. In some cases, ligands are acquired from the diet or are pharmaceutical agents. The natural ligands of LXRs are oxygenated metabolites of cholesterol (oxysterols). Some cells that synthesize oxysterols also express LXRs, making the LXR system an autocrine and paracrine system rather than a purely endocrine one.

The two major differences between TH and LXR signaling are: 1) TH governs the regulation and integration of metabolic homeostasis at the hypothalamic-pituitary level, but LXR does not; and 2) since oxysterols are not circulating hormones, LXR activation is not necessarily determined by plasma levels of oxysterols (34).

It is important to note that even classical steroid receptors can act in a paracrine manner. For example, dihydrotestosterone (DHT), a ligand for the androgen receptor, is not a circulating hormone but is synthesized from testosterone in cells expressing the enzyme steroid 5α-reductases. Similarly, 3β-Adiol (5α-androstane-3β,17β-diol), a ligand for estrogen receptor beta (35), is synthesized in cells expressing steroid 5α-reductase and 17β-hydroxysteroid dehydrogenase type 6 (36). If TH and LXR have a relationship similar to that of testosterone and DHT, the effects of TH in cells may vary depending on LXR expression.

Although LXR signaling is not regulated by the hypothalamic–pituitary–thyroid axis, LXR does regulate thyrotropin-releasing hormone (TRH). By mediating TH's action on TRH release, LXR influences

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thyroid-stimulating hormone (TSH) levels. In the absence of LXR, there is excessive TSH release, which stimulates thyroxine (T4) release from the thyroid gland. In addition, because LXR represses deiodinases, the loss of LXR can create a hyperthyroid state, which may help explain why LXR$\beta^{-/-}$ mice are resistant to obesity induced by a high-fat diet (33).

LXRs and TRs

The function of THs is mainly mediated through their binding to TRs at specific TREs (thyroid response elements) on DNA. Both TRs and LXRs bind to these response elements, which consist of direct repeats of the half-site sequence 5′-G/AGGTCA-3′, separated by four nucleotides (DR4). In the absence of their ligands, both TRs and LXRs bind to DR4, recruiting corepressors and inhibiting the transcription of responsive genes. When ligands bind, they relieve this repression by causing the release of corepressors and subsequent binding of coactivators, leading to the activation of transcription of responsive genes (37).

TRs can bind to DNA either as homodimers or as heterodimers with retinoid X receptors (RXRs), while LXRs form obligatory heterodimers with RXR (38–40). RXRs are a subgroup of the nuclear receptor superfamily, comprising isotypes α, β, and γ, which can form homodimeric and heterodimeric complexes with other nuclear receptors (41). The endogenous ligand for RXR is 9-cis retinoic acid (42). Thus, vitamin A also plays a significant role in the regulation of the immune system by TH and LXR (43).

T3, T4, and Deiodinases

T4 is a prohormone that is converted to the active hormone triiodothyronine (T3) through the action of deiodinases. The local activation of T4 to active T3 by deiodinases is a key mechanism of TH regulation of metabolism. There are two activating deiodinases, DIO1 and DIO2, and one inactivating deiodinase, DIO3 (44, 45). In humans, DIO1 is highly expressed in the liver, while DIO2 is expressed in the hypothalamus, white fat, skeletal muscle, and brown adipose tissue, where it is essential for adaptive thermogenesis (46). One key mechanism by which LXR regulates TH function in both rodents (47) and humans (48) is through the downregulation of deiodinases.

Some Complexities of LXRs and TRs Signaling in the Brain

Since cholesterol cannot cross the blood–brain barrier (BBB), it must be synthesized within the brain. Astrocytes are responsible for cholesterol synthesis, which is then transported to other cells via the transport protein apolipoprotein E (ApoE) (49, 50). Additionanally, the brain synthesizes two oxysterols: 25-hydroxycholesterol (25-HC), produced in microglia (51), and 24-hydroxycholesterol (24-HC), which is catalyzed by the enzyme CYP46A1 (cholesterol 24-hydroxylase) and expressed in neurons of the hippocampus, cortex, Purkinje cells of the cerebellum, and interneurons in the hippocampus and cerebellum (52). 24-HC is a major metabolite of cholesterol in the brain and serves as the route for excreting excess cholesterol (53, 54). Furthermore, the brain can inactivate oxysterols through CYP7B1, which catalyzes hydroxylation of oxysterols at the 6 and 7 positions (55). Although the cellular distribution of CYP7B1 has not been well investigated, it is one of the most active cytochrome P450 enzymes in the brain (56), making it very unlikely that the cells harboring this enzyme will respond to oxysterols.

T3 does not cross the BBB, but T4 does. Therefore, deiodinases are extremely important for the TH function in the brain, and defective deiodinases can lead to brain TH deficiency. TH enters the brain either directly via the BBB or indirectly via the blood–cerebrospinal fluid (CSF) barrier, with the BBB serving as the primary entry path for T4 (57). TH enters the choroid plexus through transmembrane transporters MCT8 and organic anion-transporting polypeptide 1C1 (OATP1C1) and exits the choroid plexus to enter the CSF via TH transmembrane transporters or through choroid plexus-derived transthyretin secreted into the CSF (58). DIO2 is expressed in the choroid plexus (59). LXRs regulate CSF dynamics at both the choroid plexus and the astrocytic end feet, and inactivation of LXR results in degeneration of the choroid plexus and lack of CSF in the lateral ventricles (28). This degeneration, along with the loss of DIO2, leads to reduced TH levels in the brain. Consequently, some phenotypic aspects of LXR knockout mice resemble TH deficiency. Once THs have passed BBB, their local availability depends on the activity of the astrocytic DIO2 to convert T4 to T3. T3 is subsequently inactivated in neurons by DIO3, which removes the 3′ iodine, producing 3,5-diiodothyronine (T2).

In addition to regulating deiodinases, LXRs also regulate T4 transporters. In humans, as in other primates, the BBB contains MCT8 but lacks OATP1C1 (60, 61). MCT8 is a highly specific transmembrane TH transporter responsible for the cellular influx and efflux of T4 and T3 (62). It is indespensible for driving TH-dependent oligodendrocyte differentiation and, consequently, myelination (63, 64). In humans, mutations in *SLC16A2*, the gene encoding MCT8, lead to an X-linked syndrome characterized by severe neurological impairment and altered T3 concetrations due to impaired TH uptake in the developing brain. In mice lacking both MCT8 and OATP1C1, TH concentrations in the brain are significantly affected (65).

Both TRs and LXRs bind to DR4 on DNA in the absence of their respective ligands, repreesing genes regulated by DR4 response elements. The knockout of LXR relieves repression on DR4-responsive genes; however, what cannot occur in LXR knockout mice is the activation of LXR by ligands and the recruitment of coactivators to enhance the transcription of LXR-responsive genes. To understanding the phenotype of LXR knocknout mice, we must consider both the derepression of certain genes and the absence of activation of others by LXR ligands.

TH regulates metabolic rate, body temperature, cholesterol homeostasis, and adrenergic function. Of these, only adrenergic stimulation is not shared by LXRs. TH regulates cholesterol homeostasis at two major points: it increases the low-density lipoprotein (LDL) receptor to facilitate cholesterol removal from circulation and stimulates cholesterol 7alpha-hydroxylase (CYP7A) to promote cholesterol removal from the body in the form of bile acids. LXRs act as cholesterol sensors, activated by cholesterol metabolites (1). Upon activation, they assist TH in eliminating cholesterol from the body by inducing cholesterol transporters ABCA1 and ABCG1, which transport cholesterol out of cells. However, LXRs also act at multiple levels to reduce TH function: 1) LXR reduces deiodinases, preventing the conversion of T4 to T3; 2) LXR lowers TH levels by facilitating negative feedback at the hypothalamic level; and 3) LXR induces the expression of the inducible degrader of the LDL receptor (IDOL), which decreases LDL receptor expression on the cell surface and limits LDL/cholesterol uptake (66).

LXRs and TRs in Neurodegeneration

Both LXR and TH are essential for normal brain development, influencing neurogenesis, neuronal and glial cell differentiation and migration, synaptogenesis, and myelination during early fetal life (67, 68). Dysregulation of cholesterol metabolism in the CNS has been linked to several neurological disorders (49, 69–74). Preclinical studies have indicated that LXRs and TRs can be used as targets for the treatment of neurodegenerative diseases (Figure 1), such as Alzheimer's disease (AD) , Parkinson's disease (75, 76), amyotrophic lateral sclerosis (ALS) (77), Huntington's disease, and multiple sclerosis (MS) (78).

Although these common and devastating neurodegenerative diseases are associated with aging, neurodegeneration likely begins much earlier, as disease symptoms emerge only after a significant number of neurons have already been lost. Our studies have shown marked expression of LXRβ in cortical neurons in the fetal mouse brain during later embryonic stages (19). LXRβ expression first appears in the cerebral cortex as early as E14.5 and is strongly expressed in the cortex plate from E16.5 until E18.5. After birth, LXRβ is mainly localized in cortical layers II/III. In LXR$\beta^{-/-}$ mice, there is no defect in neuronal proliferation; however, later-born neurons fail to migrate to cortical layers II/III as they do in wild-type (WT) littermates (19). This migration defect is thought to result from a defect in radial glia and reduced expression of the renin receptor, ApoER2 (79). The defect is corrected when TH levels increase, and by postnatal day 14, there is no detectable difference in the cortex between WT and LXR$\beta^{-/-}$ mice (79). These observations suggest that in the absence of LXRβ, there is insufficient TH in the fetal brain, leading to a prolonged repressive role of TR- on TH-responsive genes.

Despite the well-known effects of TH in the developing brain and the clear role of fetal hypothyroidism in mental retardation, TH is not implicated in late-onset neurodegenerative diseases. However, the loss of

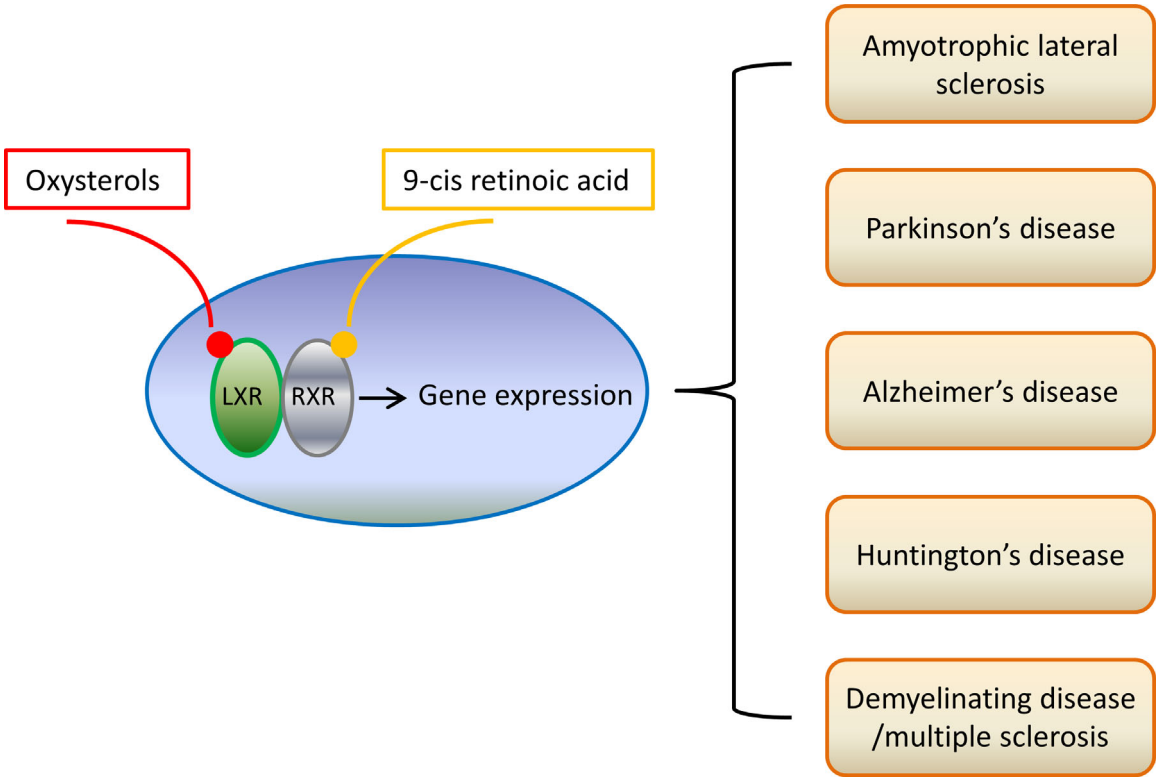

Figure 1. LXR and related CNS neurological disorders.

LXRβ in mice does lead to age-related neurodegeneration. In LXR$\beta^{-/-}$ mice, there is a loss of dopaminergic (DA) neurons in the substantia nigra (80), large motor neurons in the ventral horn of the spinal cord (81), epithelial cells of the choroid plexus (28), retinal ganglion cells (15), and spiral ganglion neurons (Figure 2) (16). All of these conditions develop with age after the mice are 6 months of age.

One perplexing observation, in view of the loss of DA and motor neurons, is the absence of LXRβ expression in these neurons in adult mice. This has led to the conclusion that LXRβ in cells other than DA and motor neurons protects these neurons from age-related loss. These specific cell types involved remain to be identified. To date, LXRβ has been specifically deleted from astrocytes (82) and microglia, and there was no observed loss of DA or motor neurons in these mice. It remains possible that degeneration of the choroid plexus and defects in CSF, which occur in the absence of LXR, are major contributors to the neurodegeneration observed in LXR$\beta^{-/-}$ mice.

LXRs and TRs in ALS

ALS is a late-onset, fatal neurodegenerative disorder characterized by the specific loss of both upper and lower motor neurons (83). The majority of cases are classified as sporadic, with the etiology remaining unknown. Less than 10% of ALS cases are familial and associated with defects in the *SOD1*, *C9ORF72*, *FUS*, and *TARDBP* genes. Although none of these genes are regulated by LXR, a proteomic analysis of serum from ALS patients revealed that the LXR/RXR pathway is one of the most significantly regulated pathways, with both LXRα and LXRβ identified as genetic modulators of the ALS phenotype (84, 85). In mice lacking LXRβ, there is progressive impairment of motor performance leading to hind limb paralysis, loss of motor neurons in the ventral horn of the spinal cord (Figure 3), and loss of neuromuscular junctions (80, 81, 86).

A study on the pathogenesis of ALS indicated that 25-HC, an endogenous ligand for LXR, may actively mediate neuronal apoptosis, particularly in the early symptomatic stage of the disease (87). The failure of the CNS to remove excess cholesterol can lead to neurodegeneration, as the accumulation of cholesterol may be toxic to neuronal cells. However, cholesterol accumulation is not the only brain defect caused by defective LXR; there is also a reduction in 3β,7α-dihydroxycholest-5-en-26-oic acid, a neuroprotective cholesterol metabolite (88, 89).

Another common defect observed in both ALS and LXR$\beta^{-/-}$ mice is the structural and functional disruption of the blood–CSF barrier. In ALS, there is disruption of junctions between choroid plexus epithelial cells, activation of platelets, immune infiltration into the choroid plexus, and degeneration of major vasculature associated with the disease (90). The choroid plexus of LXR knockout mice is severely affected (28), with degeneration and absence of CSF in the lateral ventricles being prominent characteristics of the LXR$^{-/-}$ mouse brain.

To date, studies have not provided strong evidence to support a role for TH in ALS. In a cohort of Portuguese patients with ALS, thyroid dysfunction was not associated with the disease (91), and in a cohort from Southwest China, thyroid dysfunction did not associated with survival or serve as a prognostic factor for ALS (92). Despite the lack of effect of TH on ALS, a similar movement disorder is observed in both TH and LXR deficiencies: pronounced, spontaneous, asymmetrical circling behavior. This behavior was first reported by Kincaid (93) in genetically hypothyroid mice, which does not develop a thyroid gland due to a defective TSH gene. The circling behavior appeared in both male and female mice around postnatal day 35 and persisted throughout their lifespan. The circling was unidirectional, either clockwise or counterclockwise. This behavior was noted in all female but not male LXR$\beta^{-/-}$ mice. In the Kincaid study, the cycling was linked to the loss of DA neurons in the substantia nigra, but it remains unclear why the behavior only emerged in adult mice. In the LXR$\beta^{-/-}$ mice, a similar cycling behavior was observed, though its etiology has not been thoroughly investigated.

LXRs and TRs in Dopaminergic Neurons

In the developing mouse brain at E11.5, the LXR agonist 24(S),25-epoxycholesterol increased midbrain DA neurogenesis from precursor cells by about 40% *in vitro* and *in vivo* (94–96). The LXR-regulated transcription factor responsible for this increase in the differentiation of radial glia into DA neurons was identified as the basic helix-loop-helix

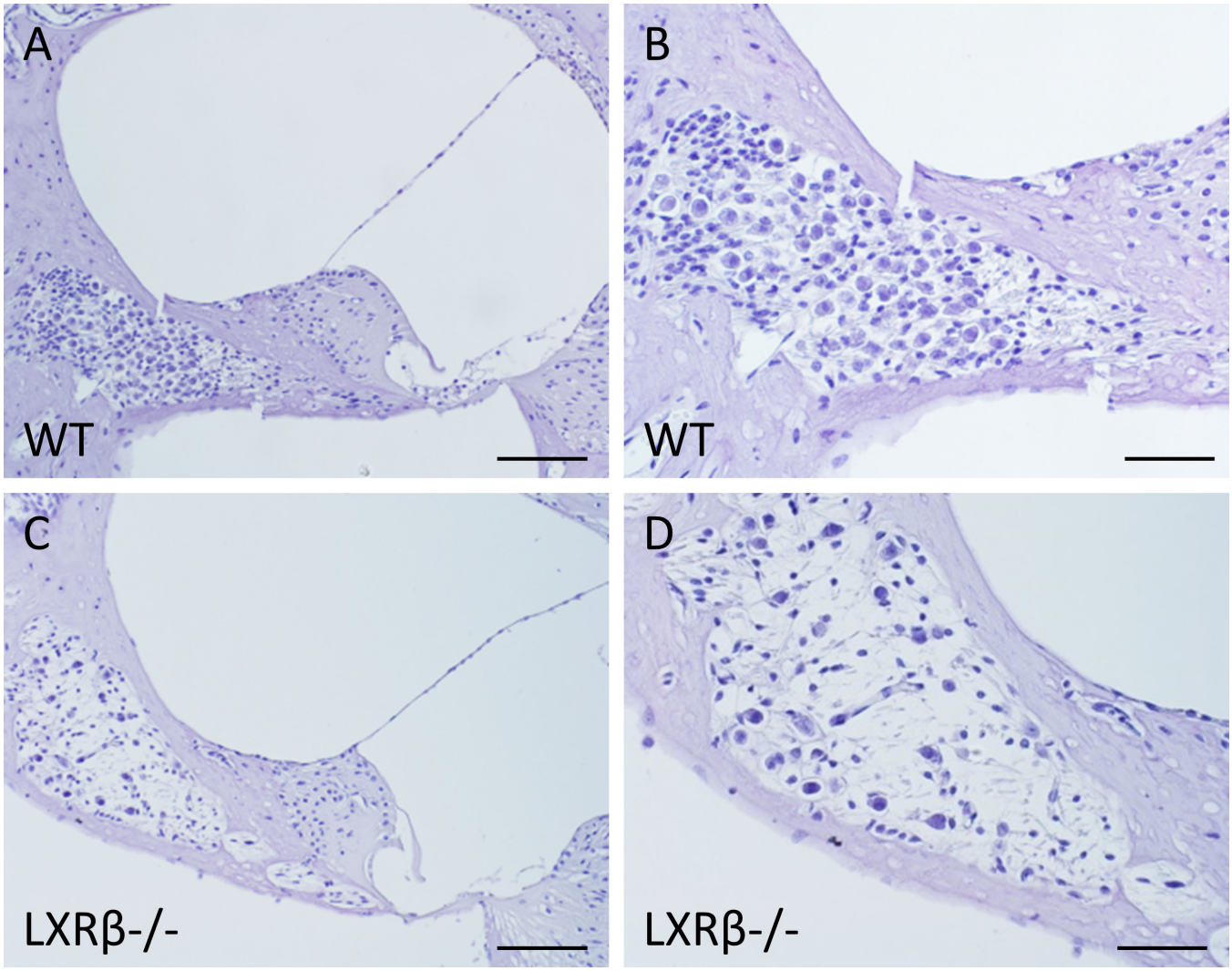

Figure 2. The number of spiral ganglion neurons in the cochlea of LXR$\beta^{-/-}$ mice is less than that of WT mice. 12 months of age. Scale bars: A and C, 100 μm; B and D, 50 μm.

transcription factor SREBP1 (sterol regulatory element binding protein 1; gene name *Srebf1*) (97). Despite this role of LXRβ in the differentiation of DA neurons, there is no apparent reduction in the number of DA neurons in the substantia nigra in 5-month-old LXR$\beta^{-/-}$ mice, and their performance on the rotarod test was comparable to that of WT mice (80). Thus, there is a disconnect between LXR's actions in the fetal development of DA neurons *in vitro* and its role in the adult brain.

Knocking out LXR affects the survival of DA neurons. In the substantia nigra of LXR$\beta^{-/-}$ mice, the loss of DA neurons begins to be noticeable after the mice reach 6 months of age, and by 16 months, there is a marked reduction in the number of DA neurons. These mice perform poorly on the rotarod. LXRβ knockout mice also show increased sensitivity to challenges with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (17) or β-sitosterol (80). A confounding factor in these effects is that LXRβ is not expressed in DA neurons. Thus, LXR appears to influence DA neurons at two levels: survival with age and neurogenesis at E11.5. In both cases, it is not LXRβ in the DA neurons themselves, but in other cells that influence the differentiation of DA neurons. The cells involved during embryonic development are likely radial glia, but the specific cells responsible for the loss of DA neurons in adult mice with age remain to be determined. It may be that multiple LXR-regulated cells and factors, including cholesterol accumulation, microglial activation, astrogliosis, or a dysfunction of the choroid plexus, influence the survival of DA neurons.

TH is also essential for the differentiation of DA neurons (98), but in this context, it is evident that TRα1 in precursor cells, rather than in DA neurons, is responsible (99). The transcription factor required for embryonic ventral midbrain neural stem cells (NSCs) to differentiate into DA neurons is Otx2. TRα1 is coexpressed with Otx2 in cultured ventral midbrain NSCs. Otx2, in turn, induces a number of other factors, including Neurogenin 2 (Ngn2) and Nurr1 (also known as nuclear receptor 4A2, NR4A2).

Currently, the distinct roles of LXR and TH have not been fully defined. Published data indicate that the functions of TH and LXR have been investigated at different stages of differentiation between E11.5 and E13.5. This is a critical period for the differentiation of DA neurons (100), and many steps in DA neuronal differentiation occur before E13.5. Until more detailed timed studies are conducted, it is not possible to separate the roles of TH and LXR in the differentiation of DA neurons.

Of key interest to human disease is the late-onset of loss of DA neurons in LXR$\beta^{-/-}$ mice. Since Parkinson's disease is a late-onset condition, the LXR$\beta^{-/-}$ mice may provide valuable insights into this disease.

TRs in Cerebellum

Ishii *et al*. summarized that various mouse models have been used to evaluate the effects of TH on cerebellar development, reveling extensive abnormalities that result in an ataxic phenotype (101). The postnatal

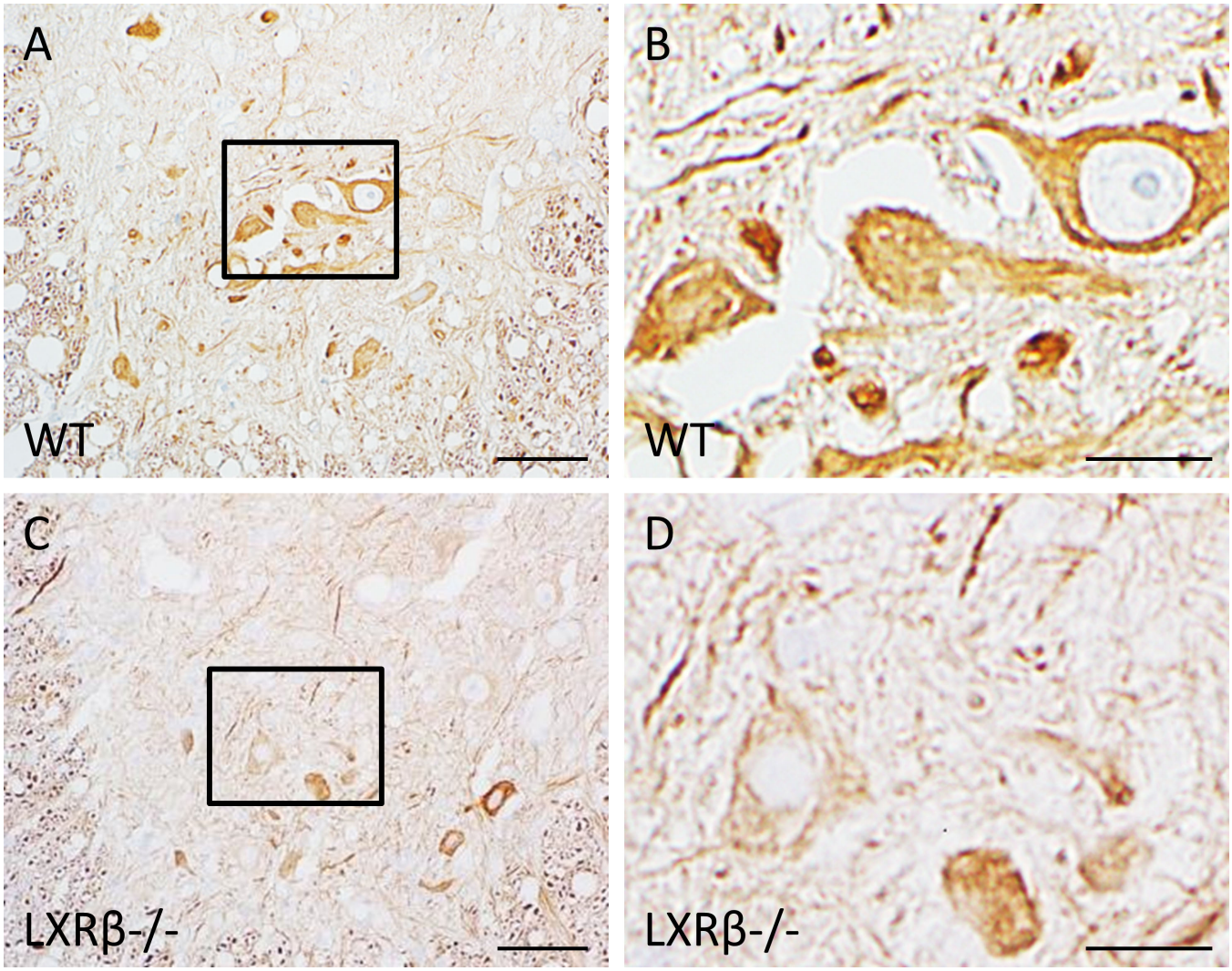

Figure 3. The number of motor neurons in the ventral horn of the spinal cord of LXR$\beta^{-/-}$ mice is less than that of WT mice. Neurofilament staining. 11 months of age. Scale bars: A and C, 50 μm; B and D, 20 μm.

defects observed in the cerebellum of hypothyroid mice are recapitulated in mice heterozygous for a dominant-negative mutation in the TRα1 receptor (102, 103). This mutation primarily affects the differentiation of Purkinje cells and Bergmann glia.

LXRs and TRs in Development of the Dentate gyrus

The dentate gyrus (DG) of the hippocampus plays a prominent role in learning, memory, and emotion. The subgranular zone (SGZ) of the hippocampal DG is one of the stem cell–containing niches in the adult mammalian brain (104). The permissive milieu of the SGZ allows NSCs to proliferate while promoting the specification and differentiation of dentate granule neurons. In the DG of LXR$\beta^{-/-}$ mice, there is hypoplasia and abnormalities in progenitor cell formation and granule cell differentiation, resulting in autistic-like behavior (105). In GW3965-treated 3xTg-AD mice, the number of stem cells and proliferating cells increased in the SGZ (106). Furthermore, LXR activation ameliorated learning and memory impairments by promoting neuronal survival, NSC proliferation, and neurogenesis in the DG in different animal models (107, 108).

Hypothyroidism results in reduced hippocampal volume in adults (109). THs affect neurogenesis in the DG of adult rats (110) and are essential for preserving nonproliferative cells involved in adult neurogenesis (111). In 2024, Valcárcel-Hernández *et al*. provided an excellent summary of THs in the SVZ (subventricular zone) lining the lateral ventricles, the hippocampal SGZ, and the hypothalamus, controlling the generation of new neuronal and glial progenitors from NSCs, as well as their final differentiation and maturation programs (112).

LXRs and TRs in Alzheimer's Disease

AD, the most common cause of dementia globally, is a progressive neurodegenerative disease characterized by initial memory impairment and cognitive decline, with the presence of amyloid plaques and neurofibrillary tangles being crucial for a pathological diagnosis (113). Both TH and LXR signaling have been implicated in AD (Figure 4). As discussed above, LXR signaling is intricately linked to TH levels. Because LXR inhibits deiodinases, a reduction in LXR signaling should be associated with higher levels of TH. Therefore, the reduced signaling of both TH and LXR in AD is puzzling.

Several studies have investigated the association between thyroid dysfunction and dementia risk (114–116). Meta-analyses revealed a higher prevalence of hypothyroidism in AD, but the authors cautioned that the finding could not distinguish whether hypothyroidism is a risk factor for or a consequence of AD (117). One of the most beneficial effects of TH in AD is its effects in repression of microglial immune responses (118). However, no definitive link between thyroid dysfunction and AD has been established (119–121).

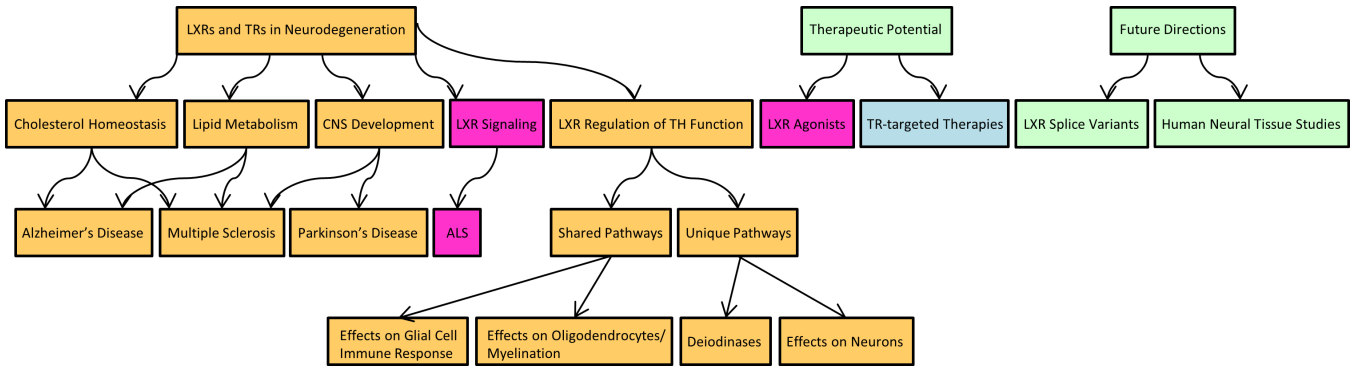

Figure 4. Unraveling the complex roles of LXRs and TRs in neurodegenerative diseases. This diagram depicts the intricate biology of LXRs and TRs and their roles in neurodegenerative diseases. At the heart of our conceptual framework is how these receptors influence key processes in the brain—from managing cholesterol levels to shaping brain development. We can see how their actions ripple out to affect various neurodegenerative conditions, including Alzheimer's and Parkinson's diseases, as well as ALS and multiple sclerosis. An interesting twist revealed by the diagram is that LXRs actually help regulate thyroid hormone function, adding another layer of complexity. We have used different colors to highlight which processes are specific to LXRs (in pink) or TRs (in light blue), while shared pathways are shown in orange. Looking to the future, we have included promising therapeutic approaches and exciting new research directions in light green. This visual framework captures our current understanding and also points to where the next chapters in this emerging scientific narrative might lead us.

In the context of LXR and AD, activation of LXR has been considered a therapeutic strategy (11, 71, 73, 122–124) for several reasons: 1) ApoE, an LXR-induced gene, promotes the proteolytic degradation of Aβ in various AD animal models (106, 125–128), thereby reducing brain Aβ burden; 2) Inhibition of neuroinflammation (129, 130), including the activation of microglia and astrocytes (131, 132); 3) LXR ligands ameliorate the impairments in synaptic plasticity (133, 134); 4) Genetic loss of LXRs in APP/PS1 transgenic mice results in increased amyloid plaque burden (135); 5) T0901317 has beneficial effects on memory by enhancing brain cholesterol turnover in APPSLxPS1mut mice (136); 6) In APP/PS1 mice, LXR agonists exert beneficial effects in ameliorating memory impairment by elevating levels of ApoE and ABCA1, reducing the expression of proinflammatory genes, and decreasing Aβ aggregation (137–139); 7) Activation of LXR with the agonist T0901317 decreased BACE1 expression and activity by lowering membrane cholesterol levels (140); 8) DMHCA, a partial LXR agonist, prevented memory decline and significantly decreased hippocampal Aβ oligomers without affecting plasma lipid levels (141).

One gene that is upregulated by both LXR and TRβ is the seladin-1 (selective AD indicator-1), encoded by the 3beta-hydroxysterol-Delta24 reductase (DHCR24). DHCR24 is a crucial enzyme in cholesterol synthesis, catalyzing the conversion of desmosterol into cholesterol and lanosterol to 24,25-dihydrolanosterol. Both LXRα and TRβ upregulate the transcription of DHCR24 (142–144), suggesting it may be a common gene linking TR and LXR to AD.

LXRs and TRs in Demyelinating Diseases

Since cholesterol is an essential component of all cell membranes and is particularly enriched in myelin membranes, it is not surprising that cholesterol metabolism is involved in the processes of demyelination and remyelination (145). Oligodendrocytes are the cells in the brain responsible for myelination (146). Both LXRs and TRs are critical for promoting and maintaining myelination (147, 148). Even before myelin synthesis occurs, both receptors are needed for the differentiation of oligodendrocytes. LXRβ regulates the number of oligodendrocyte by driving radial glial cells in the dorsal cortex to become oligodendrocyte progenitor cells (149). Meanwhile, TH is required for the terminal differentiation of oligodendrocyte precursor cells into myelinating oligodendrocytes by inducing rapid cell-cycle arrest and transcription of prodifferentiation genes (150, 151).

Therefore, it is not surprising that the knockout of LXRs in mice results in abnormal myelination and a reduction in the size of myelinated axon in the mouse brain (70, 152, 153). As described above, LXR has widespread functions in the body, and inactivation of LXR leads to multiple organ dysfunction in mice. If LXRs have similar roles in humans and mice, it is difficult to imagine a human surviving with a defective LXR gene without severe defects in lipid homeostasis, vascular disease, and immune and neuronal dysfunction. A mutation in LXRα (p.Arg415Gln) has been reported to be responsible for familial developing progressive MS (154), but the association between the LXRα mutation and MS could not be confirmed by the International MS Genetics Consortium (IMSGC) patient collection (155). Before this issue can be fully resolved, it is essential to examine the function of the LXRα with the (p.Arg415Gln) mutation to determine whether it functions as a normal LXRα and whether the LXR mutation simply segregates with another gene responsible for the MS phenotype.

Martin-Gutierrez *et al.* reported that LXR-mediated lipid metabolism pathways were dysregulated in T cells from patients with relapsing-remitting MS (RRMS) pathology, potentially contributing to RRMS pathogenesis (156). The study shows that LXR regulates T cell function by regulating glycosphingolipid and cholesterol metabolism, although the specific defect in LXR in T cells that could cause RRMS remains undefined.

MS is an autoimmune disease (78, 157, 158) thought to be due to T-cell reactions to antigens associated with myelin, such as myelin basic protein and myelin oligodendrocyte glycoprotein. In chronic demyelinating inflammatory disease models, TH restores normal levels of myelin basic protein mRNA and protein (159, 160) and promotes the differentiation of oligodendrocyte progenitor cells, improving remyelination through TRβ-mediated T3 effects (161).

T4 activates oligodendrocyte precursors and increases the content of myelin-forming proteins and NGF in the spinal cord during experimental allergic encephalomyelitis (162). Studies using the TRβ-selective agonist Sobetirome (GC-1) have found that it promotes remyelination, enhances oligodendrocyte proliferation, and protects against oligodendrocyte death (163–166).

In addition to its effects on oligodendrocytes, another mechanism through which LXR signaling repairs demyelination damage is by acting on microglia/macrophages, inhibiting the inflammatory response and providing a supportive environment for oligodendrocyte differentiation and myelination (167), while also promoting the phagocytic clearance of myelin debris and cholesterol (168). LXR agonists may be useful in healing white matter injury, as LXR ligands have been shown to induce oligodendrogenesis in rodent injury models (169, 170). However, the challenge of limiting LXR action to the targeted area must first be addressed.

Concluding Remarks

The aim of this review was to analyze the roles of LXRs and TRs in neurodegenerative diseases (Figure 4). A review of the literature clearly shows that these two receptors work together to regulate cholesterol homeostasis, and dysregulation of cholesterol homeostasis is a common factor in neurodegenerative diseases. Due to their widespread effects

throughout the body, it is unlikely that generalized dysfunction of either receptor would lead to selective degeneration of certain neurons without causing other significant defects in the body. One possibility that has not yet been addressed is the existence of LXR splice variants that are selectively expressed in the CNS, and it may be dysregulation of these splice variants that contributes to neurodegenerative diseases. Multiple splice variants of both LXRα and LXRβ have been reported (171), but their roles in disease have not yet been investigated. Additionally, the differences in the genomic and physiological functions of nuclear receptors between humans and rodents cannot be ignored, highlighting the need for more research on nuclear receptor signaling in humans or nonhuman primate. In conclusion, it will be crucial to study nuclear receptors, including LXRs and TRs, by investigating their splice variants and examining neural tissues from patients with neurodegenerative diseases.

Author Disclosures

The authors declare no conflicts of interest.

Author Contributions

J-ÅG, MW, and XS conceived the manuscript topic, edited and organized the final draft. XS wrote the first draft of the manuscript and prepared the figures. All authors revised the final manuscript and approved the final version.

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THOUGHT LEADERS INVITED REVIEW

Lessons we learned from the Lothian Birth Cohorts of 1921 and 1936

Ian J. Deary[1], and Simon R. Cox[1]

The authors are, respectively, the founding and current Directors of the Lothian Birth Cohorts of 1921 and 1936. In this invited and, admittedly, self-regarding and necessarily self-citing piece, we enumerate and explicate some things we learned from working with the cohorts and their data. Some of the lessons are scientific results, some are to do with scientific practice, and some are more general reflections. We hope the paper provides a useful summary of some of the main findings from these too-many-papers-to-read cohorts and an enjoyable account of our building a research team and a network of collaborators. The original aim of assembling the cohorts was to fashion a tool to discover why some people's thinking skills aged better than others'. That tool, we discovered, had many additional uses.

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Keywords: Cognitive ageing, cognitive epidemiology, longitudinal studies, Lothian Birth Cohorts, Scottish Mental Surveys, intelligence.

Introduction

It is nice (we are British, after all) to have been asked by the editor to reflect on what we have learned from the Lothian Birth Cohorts (LBCs). We are happy to do so. One of the benefits this reflection affords is that it can collect a fraction of the large number of the LBCs' widely-dispersed scientific articles in one place and provide a shop window for them (see Publications from the Lothian Birth Cohorts); it can point the way to many more. There are some drawbacks, too, of this exercise. We shall necessarily focus on the LBCs' contributions to scientific questions whereas we know that other cohorts and samples often have made more and better contributions. We shall have to engage in the frowned-upon activity of self-citation. We try to avoid duplicating other synoptic pieces on the LBCs. These coy worries notwithstanding, here are some lessons from 25 years of work on the LBCs.

Not everyone knows what the LBCs are, so this enumerated paragraph is a crib sheet. Here are some key facts that should make the rest of the article more comprehensible.

1. On Monday June 1, 1932, the Scottish Council for Research in Education tested almost every child born in 1921 and attending schools in Scotland on the Moray House Test No. 12 (a test of intelligence that correlated about 0.8 with the Stanford Binet test in 1000 of the pupils in a validation exercise). The *N* was 87,498 and this represented about 94% of the whole Scottish population of that year of birth. This was the Scottish Mental Survey 1932 (SMS1932) (1, 2).
2. On Wednesday June 4, 1947, the Scottish Council for Research in Education tested almost every child born in 1936 and attending schools in Scotland on the Moray House Test No. 12. The *N* was 70,805 and this represented about 94% of the whole population of that year of birth. This was the Scottish Mental Survey 1947 (SMS1947) (2, 3).
3. Beginning in 1999, at a mean age of 79 years, we recruited 550 largely-healthy community-dwelling Scottish people born in 1921 to form the Lothian Birth Cohort 1921 (4). Most had taken part in the SMS1932; therefore, for most of them, Moray House Test scores were available from age 11. They provided demographic and health information; they were tested on cognitive functions, sensory functions, psychosocial factors, and fitness; they provided blood samples for a wide range of biomarkers, genetics, and other 'omics tests; they were linked to death records; a minority had some structural magnetic resonance imaging (MRI) of the brain. They were tested at ages 79, 82, 87, 90, and 92 years (5, 6).
4. Beginning in 2004, we recruited 1091 largely-healthy community-dwelling Scottish people born in 1936 to form the LBC1936 (7). Most had taken part in the SMS1947; therefore, for most of them, Moray House Test scores were available from age 11. They provided all the information that has been collected in the LBC1921, but in more detail and with many extras. For example, their cognitive test battery was much longer, they underwent longitudinal structural magnetic resonance brain imaging, they were linked to medical records as well as death records, they provided white blood cells for stem cell creation, and they consented to provide brain tissue after death. They were tested at ages 70, 73, 76, 79, 82, 86 and, as we write, they are being tested for what will comprise Wave 7 at mean age 88 (5, 6). Figure 1 illustrates the timeline of the LBC1936 study and some of the major types of data that have been collected.
5. We have written the protocols of the LBC1921 and LBC1936 baseline Waves (4, 7), and we have written cohort profiles (5) and cohort profile updates (6) that give details of the variables collected in these two studies. We recommend these articles to those who would like to request data to test their hypotheses on the LBCs.
6. We wrote a summary of what we had found out about healthy cognitive ageing in the LBC1921 and LBC1936 up to 2018 (8).
7. For those interested in the background to the LBCs, the Scottish Mental Surveys, and the smaller but slightly earlier-conducted Aberdeen Birth Cohorts of 1921 (ABC1921) and 1936 (ABC1936) there is the book, *A Lifetime of Intelligence* (2).
8. A key variable that is available in the LBC1921 and the LBC1936 was the retesting in old age of the Moray House Test No. 12. This is the intelligence test that they had taken at mean age 11 years, which was the age of transition from primary to secondary school (at the time, compulsory education continued until the age of 14).
9. Ian Deary founded and directed the LBCs from January 1999 to November 2020 when he retired (just briefly, to be rehired, a few months later, part-time to continue working on the LBCs). Simon Cox has Directed the LBCs since December 2020, having worked with the LBCs since 2009 (for his PhD where Ian Deary was one of his supervisors, then as Study Co-ordinator, then postdoctoral fellow, and then LBC Co-Investigator and leading his own funded work on the neuroimaging aspects of the study).
10. It was a fumbling set of events that led Lawrence Whalley (who died in 2024) and Ian Deary to discover that the SMSs had been conducted and that their data still existed (described in ref. 2). Professor

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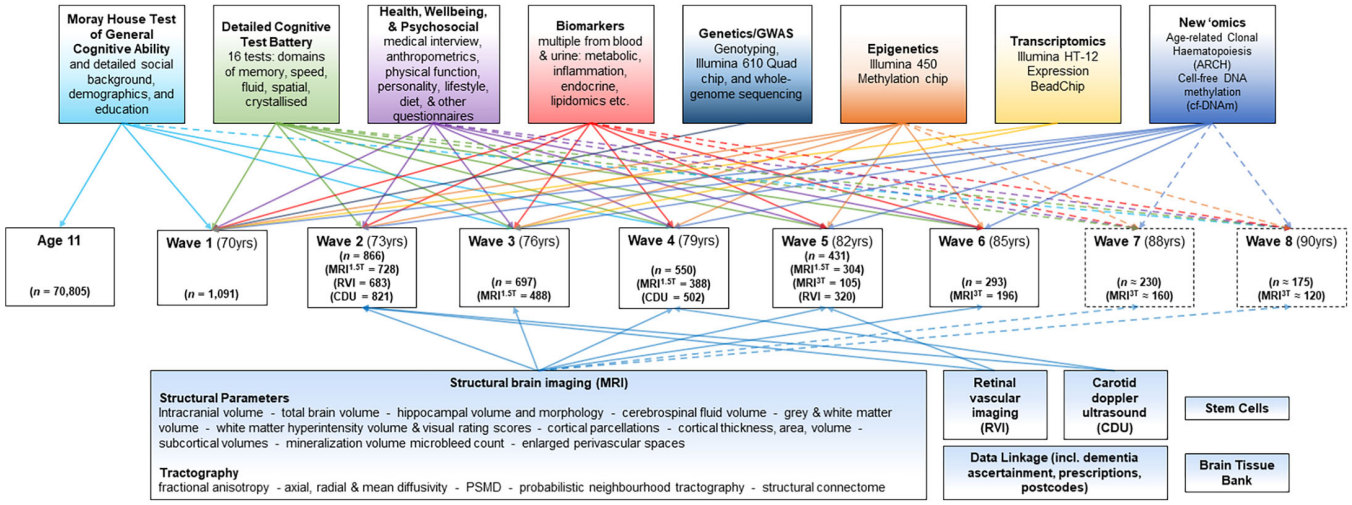

Figure 1. Data collected in the Lothian Birth Cohort 1936. The central row of white boxes denotes each instance of measurement, starting with all age 11 Scottish children who sat the Moray House Test Number 12, and proceeding to the "baseline" (Wave 1) and subsequent assessment visits of the LBC1936 participants. Solid arrows indicate data (top and bottom rows) collected at a given wave; dotted lines and boxes denote ongoing (Wave 7) and planned (Wave 8) data collection.

Whalley (a psychiatrist) led the ABC1921 and ABC1936, collaborating with Ian Deary and geriatric physician Professor John Starr (9). John Starr was the medical lead on the LBCs from 1999 until his death in 2018, after which Dr Tom Russ took up the role. Professor Joanna Wardlaw (a neuroradiologist) is the brain imaging lead on the LBC1936 (10).

Scientific Discovery Lessons

When people ask about the aims of the LBCs, we say something like, "we are trying to discover why some people's thinking skills and brains age better than others". However, the LBCs have proved to be valuable far beyond that remit. They often then ask, "what have you discovered?". After we provide an answer, sometimes it is met with undertakings to make lifestyle changes (e.g., stop smoking), but it is also not unusual to hear the follow-on question, "isn't that obvious?". See what you think...

Some of the Big Findings Appeared Early on

This is not a scientific discovery per se; rather, it is a meta comment. To articulate this, although it has a Pareto-like quality (and some regression-to-the-mean quality), we shall use the comparison of pop/rock bands. Most bands have many songs, only a few of which are large hits and often those hits appear early in their careers (have a look at numbers of plays on Spotify). With the Aberdeen Birth Cohorts (ABCs) and LBCs some of the relatively bigger discoveries happened early on as we picked some low-hanging fruits. Perhaps, with most cohort studies more generally, investigators will have, probably, only a few big hits and many worthy album tracks (have a look at numbers of citations on Google Scholar). Slightly to argue against that, is that longitudinal cohorts gain value from having more waves and, therefore, some larger findings can only appear after several waves of testing, not early on. In what follows we shall provide an as-pithy-as-we-can-manage statement of some of what we found, followed by a bit of explanation and context, and relevant references. There are many hundreds of peer-reviewed articles that analyze LBCs' data and we shall cite, in total, a small minority of them.

Higher Intelligence Test Scores at Age 11 are Related to a Better Chance of Survival to Older Age, and to Lower Risk of Death From Many Major Causes of Mortality

These—the associations between higher childhood (sometimes young adulthood) intelligence test scores and longer life and better health—have been widely replicated, including in very large studies (some having six or seven figure sample sizes). The discovery from the SMSs, that higher intelligence in childhood is associated with living longer (11, 12), properly began the field of cognitive epidemiology which aims to replicate, extend, and explain this set of findings (13). Figure 2 shows the results of linking the Moray House Test scores at age 11 from the Scottish Mental Survey 1947 to major causes of death several decades later. This new field took we psychometrically-oriented psychologists into the statistical analysis world and tools of epidemiologists. The association between childhood cognitive test scores and survival was analyzed mostly using Cox proportional hazard regression and the results expressed as hazard ratios. To give a guide to the size of the typical effects, a one-standard deviation advantage in Moray House Test score at age 11 was associated on average with about 20% to 25% lower chance of dying from most major causes of death up to the late 70s (12). Part of this work has been the picking-apart of the contributions (confounders?, mediators?) of education and social class (which are correlated with intelligence test scores and are themselves related to health and mortality inequalities), and the employing of molecular genetic techniques. We have reviewed this field and, briefly, it appears that childhood (parental) social class does not contribute much if at all to the intelligence-longevity/health association, but that the association might be mediated somewhat by a person's own adult social class (14). With regard to education (or, e.g., health literacy) this is hard to call, not least because intelligence and education and health literacy are quite strongly correlated (15).

About Half the Variance in Intelligence Test Scores in Older Age is the Same as That Found at Age 11

Not long after we discovered that the SMSs' data were extant, we knew that it would be valuable and unusual to be able to find out how strongly childhood intelligence test scores correlated with the same test taken in older age. This provides two useful pieces of information: the obverse is the stability of intelligence differences across most of the human life course (tested using the Pearson [usually] correlation between the test score at age 11 versus the score on the same test in older age); and the reverse of that coin is that it can tell us about the changes with respect to individual differences over that same period. For the former, we've published several papers that describe the correlation between the Moray House Test No. 12 at age 11 and older-age ages in the 60s, 70s, 80s, and 90s (4, 16–18). The broad result is that even the raw correlation from age 11 to the 70s is not far from 0.7 which, when squared, tells us that just under half of the variance in intelligence in older age was there at age 11. This is a lower-bound estimate of the long-term stability of intelligence differences. It is not corrected for measurement error or for the restriction of range in these samples compared with their background populations (which are known because of the comprehensiveness of the Scottish Mental Surveys) (18). For the latter (i.e., the remaining ~50% not explained by early life differences, some of which will be measurement error, of course), understanding what sorts of factors (be they genetic, health, behavioral,

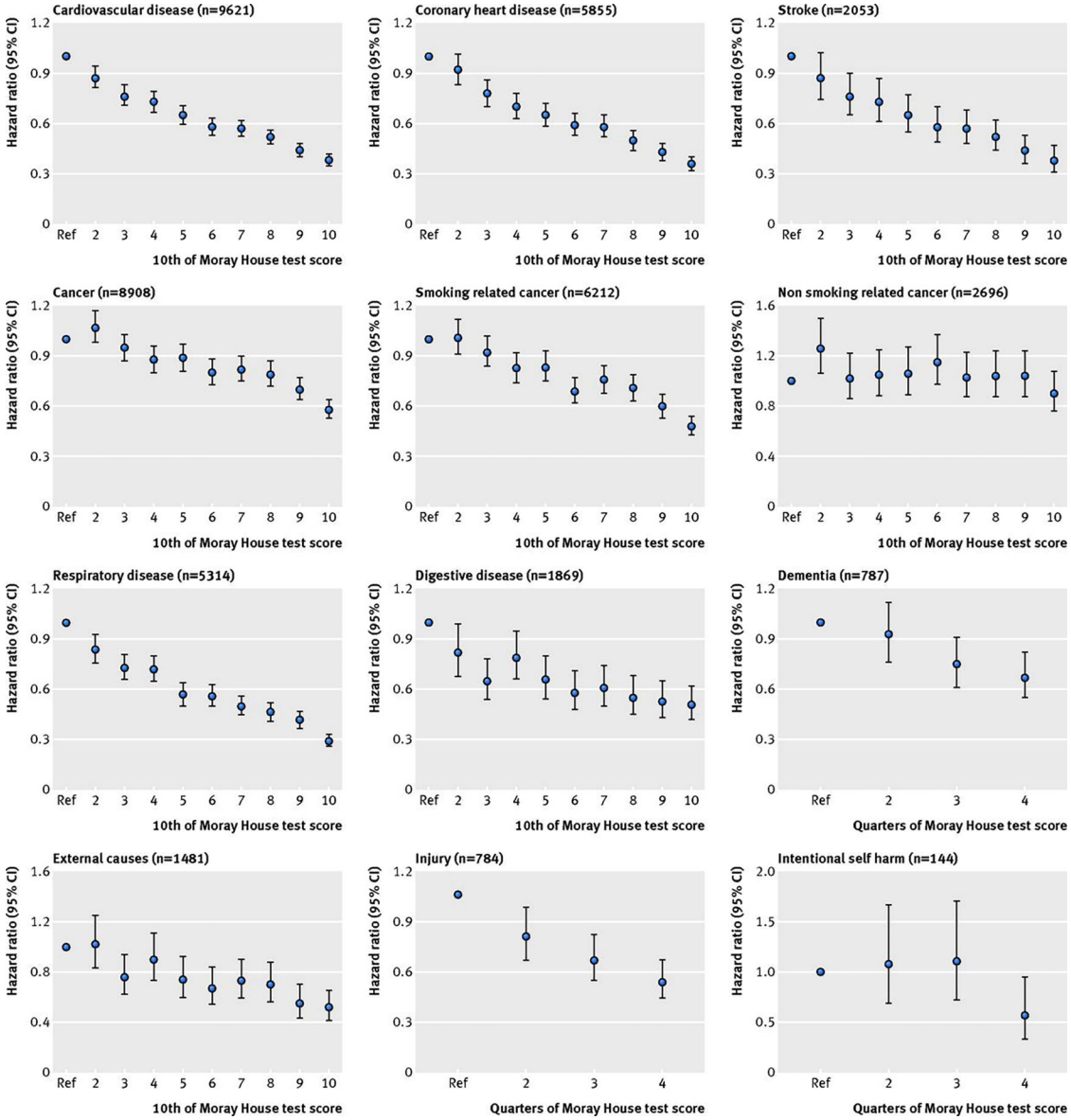

Figure 2. Association between Moray House Test No. 12 score at age 11 and major causes of death up to age 79 in the Scottish Mental Survey 1947. For visualization, the Moray House Test scores were divided into deciles or quarters. The points in the figures are age- and sex-adjusted hazard ratios and 95% confidence intervals; the lowest scoring group is set to 1.0. The analytic sample *N* was 67,765 of whom 25,979 had died. Mean time to follow up was 57 (SD = 18) years. This is Figure 2 from Calvin et al. (2017) in the British Medical Journal, 357, j2708; this article is an open access article distributed under the terms of the Creative Commons CC BY 4.0 license and the figure is reproduced here, with thanks, under that license.

social, etc., though some will be stochastic/random error) perturb people from their childhood ranking has been the basis of many of the earlier LBC discoveries. And it turns out that those who are perturbed less from their 11–70 score ranking are also those that tend—a bit—to also age less steeply into much older age (19). In Figure 3, we show the scattergrams of the Moray House Test No. 12 scores for the Lothian Birth Cohorts at age 11 and age 79. We have previously published a version of this scattergram for the LBC1921 but not LBC1936 and we have not published them together before. We note that, whereas age 79 was the second testing occasion for the LBC1921, it was the fourth testing occasion for the LBC1936 who also took the test at ages 70 and 76.

The Genetic Influences on Intelligence Differences are not All the Same in Childhood and Older Age

Most of the individual genetic contributions to intelligence differences are tiny (really tiny, like too tiny to work on). Don't do candidate gene studies (apart from *APOE*). So, three lessons there. The first lesson was based on an early finding with the LBC1921 in which we found that possession of the *APOE* e4 allele (assessed by testing for the two single-nucleotide polymorphisms [SNP] that determine *APOE* e4 status) was not associated with Moray House Test No. 12 score at age 11, but was associated significantly with the same test taken by the same people at age 79 (on average, those with the e4 allele scored lower) (20). The second lesson became obvious as we conducted genome-wide association studies (GWAS) which grew in sample sizes from four to five to six figures. In GWAS, one examines the association between the outcome (in this case the cognitive test scores) and hundreds of thousands of SNPs that capture genetic variation in humans (see Ref. 21) for a description of this and other genetic methods). The LBCs and ABCs formed the majority of the participants originally (22) and still contributed to the larger consortia studies (23, 24). One thing that did not change hugely as the studies grew in size was the estimated heritability of intelligence differences based on SNPs—it

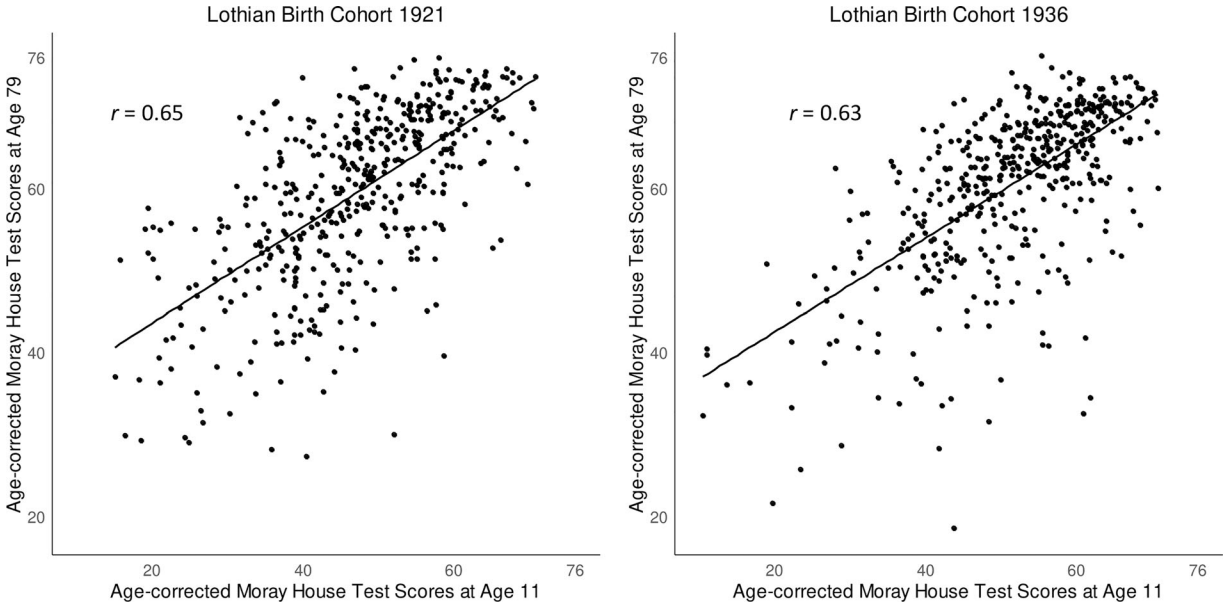

Figure 3. Stability of individual differences in intelligence from childhood to old age. Associations between Moray House Test Number 12 scores taken at age 11 and age 79 in the Lothian Birth Cohort of 1921 (left; $N = 483$) and Lothian Birth Cohort 1936 (right; $N = 468$). Pearson's r are displayed in the top left of both panels ($p < 2.2 \times 10^{-16}$). Scores have been corrected for age in days at testing. Outliers ±3.5SDs were removed from the pairwise correlations based on full available samples ($N = 3$ for LBC1921, $N = 7$ for LBC1936) for visualization purposes. Correlations with outliers included are: $r = 0.66$ (LBC1921, $N = 486$; $p < 2.2 \times 10^{-16}$) and $r = 0.61$ (LBC1936 $N = 475$; $p < 2.2 \times 10^{-16}$).

remained at about half, or a bit less, of that estimated from twin studies. That heritability, to date, is made up of at least hundreds of tiny individual associations between SNPs and intelligence test scores. We summarized this field, with consideration of what this means for understanding the biological mechanisms that found part of intelligence differences (21). The third lesson was learned from our early experience with the LBCs and ABCs and the work of others which concluded that, apart from variation in *APOE* (25, 26), associations between variation in candidate genes and intelligence test scores in modestly-sized studies have not replicated. With large errors around the point estimates, we estimated that genetic factors accounted for about two-thirds of the stability in intelligence from childhood to older age but about only a quarter of the changes in intelligence rankings across the same period of the life course (27).

Some of the Expected "Exposures" (Independent Variables) to Later-life Cognitive Ability Turn Out to be "Outcomes" (Dependent Variables) of Early-life Cognitive Ability (Reverse Causation or Confounding)

When we set up the LBCs we wanted to include as wide a range of potential contributors to people's differences in cognitive ageing as was feasible/tolerable. In testing the cognitive outcomes, we selected a broad battery of cognitive tests to cover the main domains of cognitive function; in assessing the exposures, we tried to be inclusive as was practicable and included genetic, health, fitness, sensory, biomarker, brain imaging, psychological, demographic, and social variables (6). We began to find that some of these latter, supposedly exposure/independent-variable factors, although they did associate with cognitive function in older age, also correlated with intelligence tested at age 11, many decades previously. Thus dissolved the sometimes-false separation we had made between our cognitive ageing and cognitive epidemiology investigations. Among the putative variables that were involved in our realizing this were, for example, C-reactive protein (28), physical fitness, Typical Intellectual Engagement, social and other activities (29), alcohol intake, tendency to type 2 diabetes (30) and allostatic load; there were others; some of these are listed and discussed by ref. 8. To spell this out, we found that children with a higher intelligence test score at age 11 tended to be fitter, healthier and more socially and intellectually engaged in older age, and to drink a wee bit (not a lot—not to excess) of alcohol; that is, sometimes, but not always, the association between the given factor and age-11 intelligence test could reduce the association between the factor and older-age intelligence to nonsignificance. Thus we discovered "reverse causation"/confounding by early-life intelligence test score. This does not necessarily rule out the causal nature of a given factor whose association with later life functioning is attenuated, since it could also be that people's differential exposures to cognitive-ageing-inducing factors can be predicted, at least in part, by earlier life factors. What it does do, though, is cast those factors which are not attenuated by age-11 intelligence into much sharper focus as factors of interest. The life-course timing of factors that might or might not influence people's differences in cognitive aging—including contributions made by the LBCs—is discussed by others also (31).

People Have Very Different Experiences of Brain and Cognitive Ageing

When analyzing the things that might explain differences in brain and cognitive ageing, one needs to have variability in those outcomes-of-interest. However, quite how much of a difference there is between people has been one of the striking findings of the work. The LBCs can offer a valuable window into this because all participants are the same age. The brain scans that were taken during the second wave of LBC1936 testing are a stark illustration of just how variable same-age people's brains are in terms of key features of biological ageing. Figure 4 shows a selection of 73-year-old LBC1936 brain MRIs (over 700 were brain-scanned at this age), showing atrophy (where the brain shrinks away from the intracranial vault and also the cerebrospinal fluid-filled ventricles at the center of the brain enlarge to replace space vacated by the diminishing cerebral tissue; Panel A) and white matter hyperintensities (ageing-related damage to the brain's connecting fibers; Panel B). They are both ordered from top-left (least affected) to bottom right (most affected). We and others have indicated that these and other important aspects of brain structure are important for cognitive ageing differences (see below). We have of course also shown this wide variability in the ageing experience elsewhere, with statistical figures and analyses for both brain and cognitive ageing, and for their subsequent changes into older age (which also show wide variability) (32, 33). Nevertheless, this figure remains one of the most engaging ways to communicate to others some of our central research aims; how can one arrive at older age with a brain that looks like those in the top left, and what can one do to avoid having one that looks like those toward the bottom right? And how can we maintain that for as long as possible as we

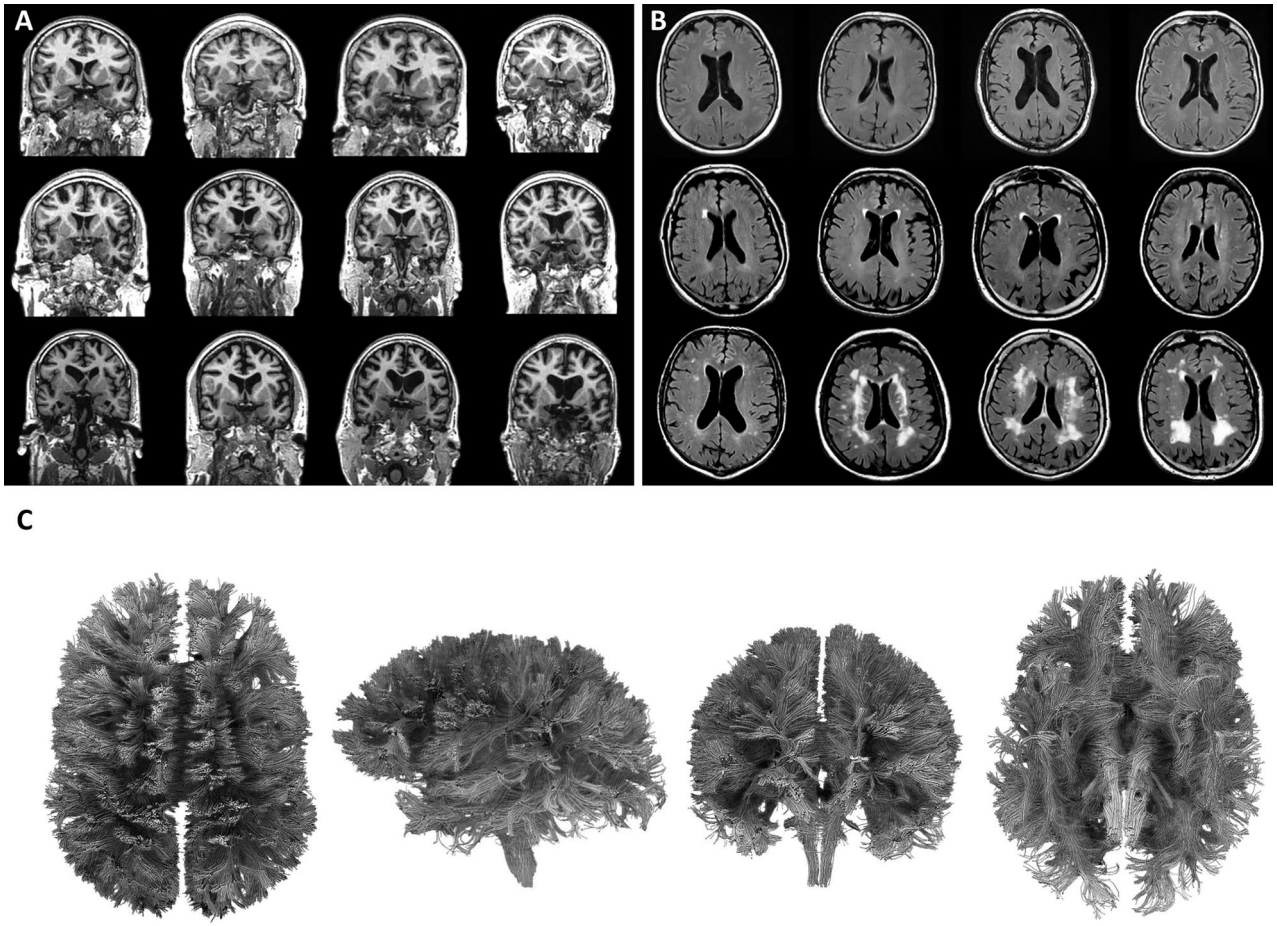

Figure 4. Brain structural (MRI) scans from a selection of individuals from the Lothian Birth Cohort 1936 taken during Wave 2 (when all participants were about 73 years old). **Panel A** shows global atrophy (brain volumetric shrinkage) ordered from least (top left) to most (bottom right). **Panel B** shows total white matter hyperintensity volume (increasing from top left to bottom right). Panels A and B are reproduced from Cox and Deary (2022) in Brain Aging, 2, 100032 (74); this article is an open access article distributed under the terms of the Creative Commons CC BY 4.0 license and the figure is reproduced here, with thanks, under that license. **Panel C** shows white matter pathways of a middle-aged male adult, identified using diffusion MRI. Views from left to right: superior, lateral, anterior, inferior.

continue to age? It also offers a ray of hope to those of us on the journey to our 70s (where IJD has just arrived) that adverse brain and cognitive ageing outcomes are not an inevitability.

Brain Size Really is (Modestly) Related to Intelligence. Whether More Intelligent People Tend to Have a Larger Brain was a Debated Issue Over Many Years

The nadir of respectability for this question might have been with Stephen J. Gould's book *The Mismeasure of Man*. The arrival of MRI to assess brain size settled the issue. Early meta-analyses, an LBC1936 study that was the largest study at the time it was published (34), and data from the UK Biobank study (35) agree that the association (correlation) between general cognitive ability and total brain volume as assessed in MRI is about 0.27. We caution that, in older-age samples such as the LBC1936 and UK Biobank, there might be sources of variation in total brain volume that are associated with intelligence test scores that are not present or as marked in younger-age samples. Therefore, it is important to study the association at different ages. Why does one do this work?: because it's the brain that thinks and we want to know how variations in its biological parameters associate with thinking skills through the life course. We recognize (see following sections) the importance in understanding what it is about a larger brain that makes, on average, for more efficient thinking. But, of course, there is much more to thinking skills than just having a large brain, including other brain variables (34, 35), and we cover some of that in the following section.

Brain White Matter Matters for Intelligence

As we just said, there is much more to thinking skills than just having a large brain. Around the time that the LBC1936 began, there was increasing realization of, and interest in, measurements of the brain's white matter and their importance for studying ageing. Our team's decision to measure participants' white matter microstructure using new diffusion MRI (see Figure 4, Panel C) was in response to this, and our intention to address this was writ large in our application to the charity Age UK for funding as "The Disconnected Mind project" (10). With the LBCs' brain imaging data, we discovered that the health of the brain's connections—the white matter—in the main brain tracts were all positively correlated, that is, healthy brain white matter in one tract was strongly related to having healthy white matter elsewhere in the brain (36). We subsequently also replicated this important finding in other healthy adult samples with a wider age range such as UK Biobank (37), and also found it in neonates and among psychiatric patients with schizophrenia (38, 39). Moreover, having computed a general component of this white matter health (using principal components analysis), we found that people's differences in brain white matter health were modestly associated with cognitive functioning (40). Thereafter, we found that these two variables change together in a synchronized fashion over time: on average, those with steeper ageing of their brain white matter pathways are those whose general cognitive functioning declines more steeply (41). This is another result that has been replicated elsewhere. Moreover, the so-called white matter lesions

that accumulate in some people more than others as they age are also related to intelligence differences and...

Brain Grey Matter Matters for Intelligence, Too (and Carefully Putting Many Brain Imaging Measures Together is Advantageous)

There are other brain variables—including grey matter parameters for the cortex and subcortex, and aspects of brain vascular health—to consider with respect to intelligence/cognitive ageing associations (34). MRI-derived variables don't stand still; we have expanded these to include detailed properties of the cortex, brain connectomics, "brain age," and other aspects of the health of the brain's white matter (33, 42–44). One lesson from this accumulation of brain imaging–derived variables is that some are strongly correlated with other such variables and that one needs to ascertain the independence of brain imaging variables from each other when firing them at intelligence differences. This avoids old-wine-in-new-bottles scenarios, but has also allowed us to: i) identify more precisely how far everyone experiences the same aspects of brain ageing; and ii) map the extent to which information gleaned from these many aspects of brain grey and white matter are all uniquely relevant for differences in cognitive ageing. We have learned that they often account for some small unique proportion of cognitive differences in older age. That is, whereas some classes of brain features are partly overlapping, having lots of information about different facets, regions, and tissues helps us to improve our understanding of differences in cognitive ageing (44, 45).

The generosity of the LBC1936 participants in providing their brain tissue after they die (more about this important and striking legacy in the sections below) has also enabled us to look deeper still into the hallmarks of better and poorer cognitive and brain ageing, identifying features like synaptic resilience and neurogranin as important aspects (46, 47). We are also using new methods to put LBC data together with large-scale postmortem data from many other sources to learn more about the regions of the brain that are most important for cognitive and cognitive ageing differences, such as gene expression patterns across the cerebral cortex (45). We are optimistic about the opportunities that these approaches promise, and we gratefully recognize that they can't happen without the sad aggregation of munificent brain donations by members of the LBC1936.

Intelligence is Far From All That Matters (at Any and Every Age as a Human)

It might be possible—given the statement of LBCs' aims given above—to imagine the LBCs as having, as outcome (dependent variable), a bullseye labelled cognitive functioning and, as exposures (independent variables), hundreds of arrows (genetic, health, lifestyle, biomarker, psychosocial, etc.) fired towards it. That would be wrong. From the beginning of the LBCs, the noncognitive variables we included sometimes became outcomes additional to the cognitive ones. We became interested in health, fitness, personality, mood, life satisfaction, social position, social engagement etc. as part of healthy ageing and studied the associations of these outcomes too (48–50).

The Age of People's DNA (by Comparison with Their Chronological Age) Predicts (to a Wee Extent) How Long People Will Live

This provides a useful lesson regarding the LBCs' expansion with respect to both exposures and outcomes. By this stage, we had already genome-wide scanned the LBCs' DNA samples for SNPs. But one's DNA nucleotide sequence is not the whole story with respect to how the DNA works (i.e., eventually leads to protein production). Along DNA strands there are, attached, methyl (CH3) groups which have effects on gene expression; these are one form of epigenetic (in this case DNA methylation: DNAm) marks. People show differences in these marks which are, in part, due to genetic differences ((51)) and, in part, to environmental causes (e.g., smoking (52)). We undertook methylome-wide scanning in the LBCs. We thought that individual differences in DNA methylation might be informative about cognitive differences and age-related cognitive changes, which they were to an extent (53). Methylation marks on DNA change with age, and we were aware of the concept of epigenetic age, that is, that some people's DNA had methylation patterns that looked older or younger than is typical for their chronological age. We found that younger methylation age at baseline (age 70 for the LBC1936 and age 79 for the LBC1921) was associated with how long people lived. This replicated in other samples. This was outside of our field but is one of our citation hits (51). Pursuant to some of the Scientific Strategy points listed below, DNAm research has been a successful and interesting collaboration. It emerged that how methylated your genes are correlated quite strongly (according to Funder and Ozer (54) rather than Cohen [see the subsection directly below]) with smoking, BMI, and inflammation, to name a few, and that these in turn are also related to brain and cognitive differences (53, 55–57).

Get Ready to Enjoy Small Effect Sizes, Moving From the Psychologists' Crud (Meehl, 1990 (58)) Value of About 0.3 Down to the Epidemiologists' 0.1 and Below

We and others saw and discovered in the LBCs for ourselves early on that effect sizes in cognitive ageing are typically small; a fitness variable, or possession of the *APOE* e4 allele, or smoking or just pick your favorite candidate variable that contributes to individual differences in cognitive ageing and about the best you can expect from any of these is that, net of cognitive capability in youth, they will, if you are lucky, contribute about 1% of the variance to cognitive capability in older age. We summarized this reality in our paper entitled *Marginal gains not magic bullet* (8). Mind you, and this seems too obvious to have to write (but we see papers and statements that refute that), it is important to keep in mind that, in cognitive ageing, what one is seeking is factors that are associated with *change* in cognitive capability, whether that change is from youth to middle to older age, or just change within older age itself when more decline takes place. To underline even more, an association between a putative "cognitive ageing" factor and a cognitive test score assessed on one occasion—whether it is cross-sectional or whether the putative predictor was assessed some time previously—is not informative about cognitive ageing. There is less variance in cognitive change than there is in cognitive status, and cognitive change is noisier, and such changes are accordingly harder to account for with predictors. Here's an example. In a 12-cohorts consortium that included LBC1936, there was a significant cross-sectional association between telomere length and various cognitive test scores (59). However, in a study that included only the LBC1936, there was no association between change in telomere length and change in cognitive test scores (or with change in physical abilities) across three waves of testing at ages 70, 73, and 76 (60). It is also worth noting that measured change is also rarer (since it is harder and more costly to measure/fund), which also detracts from the relative power of longitudinal studies on within-person differences as compared to cross-sectional studies of between-person differences (as illustrated by the 12:1 ratio in the telomere example above).

Beyond genetics—the given—what can an individual do if they want to age well, including cognitively?: play the numbers; maybe by getting oneself on the right side of the many (many of which are not confirmed) possible cognitive ageing variables one might be leaning in the right direction toward healthier ageing. Some have explained that small associations, though they can mean a lot for large populations (e.g., blood pressure control for the avoidance of stroke) are not practically informative—when considered in isolation—for individuals (61); however, we would argue again that staying on the correct/sunny side of the many small putative effects is the best choice for improving one's healthy cognitive and brain ageing (and general health) odds. With regard to the points we made in this section, and elsewhere, Walhovd et al. (31)—sometimes citing LBCs' results—made a strong case for, "sobriety regarding the timing and quantity" of influences on brain and cognitive ageing; we agree—we have tried to stay sober too, and we encourage others to do so.

Multivariate Analyses of Cognitive Ageing are More Bracing Than Univariate Analyses

Yes, it gets worse. Just as we discussed for the variety of brain imaging variables (you have to ask what each brain measure is telling you about cognitive differences that is unique), one has to ask the same about the other lifestyle, health, genetic, and other candidate predictors of cognitive ageing; that is, do they survive when entered together? There are few reliable associations with cognitive changes (usually declines, on average) in older age. And their effect sizes are small. It is not unusual for research reports to include (in addition to some sensible, basic covariates)

a single predictor of cognitive ageing. Indeed, we have had experiences of finding such reports easier to publish than when we have included multiple predictors/exposures. But life is not like that; we don't experience influences on our ageing in isolation from each other. We have conducted, with the LBC1936's longitudinal data, two studies in which we threw in a couple of handfuls of popular (from the scientific literature) determinants of cognitive ageing (32, 62). First, we looked at them as predictors of cognitive change one at a time. Then, we popped them together in a multivariate analysis. What happened?: positive findings fell like snow off a dyke (as we say in Scotland about such ephemera) as most of the significant univariate findings left possession of *APOE* e4 and the occasional other variable looking rather lonely in their continued significance. That is not to say that the unique contributions of those many factors mightn't be additively important (we examine this in this paper—(62)), but that their unique effects are likely even smaller; accurately quantifying how much smaller will require even bigger samples and consortia effort (to which we are contributing) with comparably deep phenotyping.

You Will Bet on Some Duds, But Null(-ish) Results are Valuable Too

With the LBCs we have tried to scan the horizon for possible contributors to cognitive ageing differences from many fields of study. Among these, we have kept an eye on biomarkers of ageing because that seemed like a likely source of tractable contributors. Looking back, one could say that earlier work was conducted in the time of candidate biomarkers and that we are now in a time when multi'omics platforms provide the capability to examine hundreds and even thousands of proteins/peptides, lipids, glycans, other metabolites etc. And these will be used in hypothesis-free(ish) studies and will probably deliver some replicable and probably small effects. But the point here is that we sought expert collaborators in likely biological variables related to cognitive ageing, found the resources to assess them and then sometimes found not very much when it came to looking at the results. This applies to, for example, retinal vessel topography (63), and see telomere length, above. It should quickly be said, by way of being positive, that these variables proved useful in other studies with other variables and that null results—knowing what is probably not associated with differences in cognitive ageing—provide knowledge too. One should not have an emotional reaction to a scientific result, but we confess to mild pleasure at finding a null association between childhood intelligence and life satisfaction in old age (64).

It Helps to Have a "theory" but Theory Does not Always Help Scientific Progress in This Field

We have put theory, there, in sneer quotes for the reasons that one of us has already written at length regarding the assessment of the quality of theories in the psychology of cognitive capability and most of that critique applies here (65). This lesson was learned from some referees and editors who have from time to time enjoyed our manuscripts but have wanted for some "more theory." And sometimes a nonharmful sprinkling of that condiment will suffice; we do not wish to appear cynical, but it helps to have a theory to be published, although theories in our field are often skyhooks rather than cranes (66). Some of the so-called theories that circulate in the field of cognitive ageing include brain/cognitive reserve (67, 68), brain maintenance (where others have similar reservations to ours about whether these two aforementioned "theories" constitute explanations or not (69)), common cause (70), and processing speed (71). The latter two are circumscriptions of interesting empirical regularities and the first two vary between a useful trellis on which to hang cognitive ageing studies to a diversionary soup stone (72). The one suggestion from our team that others have taken to be a theoretical articulation was the notion of "system integrity" which was posited in our first cognitive epidemiology study using data from the SMS1932 (11). We took the opportunity thereafter to clarify what it might mean and might not, and what its weaknesses and predictions were. If it deserves a name it is probably hypothesis rather than theory (73); is it a trellis or a soup stone?—neither perhaps, being more like a sticky note to remind us to explore this possibility (both theoretically and empirically) a bit more. In the field of cognitive and brain ageing we would prefer a very-large-N dataset with well-measured, relevant variables rather than an apparently "well-aimed" so-called "theory" (cf. GWAS versus candidate gene studies). We trust that this short mention of theory is not too glib, and we refer the interested reader to our longer discussions of theory (8, 65, 74, 75) and to a handbook that has a section on "models of cognitive aging" (76).

Irrespective of the Large Amount of Data you do Have, People Will (Rightly) Ask About the Data you don't Have

Running a longitudinal study of older adults inevitably results in the sad truth of dropout and missing data. Since the baseline of both LBC studies, attrition is typically about 20% per every 3-year cycle between waves of assessment. About half of the attrition is due to mortality, with the remainder being—anecdotally—a mixture of people not wishing to come back because they have "done enough," because of the development of illnesses, or being unavailable due to caring duties for grandchildren or—increasingly—for a spouse/significant other. Understanding how and why participants "drop out" of the study is important. It has important statistical implications for our core aim of characterizing cognitive and brain ageing, and asking what correlates with differences in those trajectories. We know that, on average, people who drop out are likely doing less well in terms of their brain and cognitive ageing, and general health, than those who keep coming back ("completers"; e.g., ref. 62). We also, therefore, know that, when we plot the average changes in just completers, we mostly underestimate the amount of cognitive decline in our sample (e.g., ref. 77). To ensure that we don't bias our estimates against the least healthy participants (who are just as important and informative), we will often use full information maximum likelihood (FIML) to include all available data to estimate those declines. However, reviewers often ask whether we are doing the correct thing here, since FIML assumes that the patterns of missingness are either random or mostly accounted for by variables included in our models. Whereas we are unable to account completely for the patterns of dropout we observe (e.g., refs. 32, 78), we have previously indicated that the further reduction in variance/greater range restriction in an already self-selecting sample would likely yield a slight underestimation of effect sizes (e.g., ref. 79) as well as substantially lower statistical power.

Scientific Strategy Lessons

Some of the lessons that we learned from the LBCs pertain more to how to go about the process of scientific enquiry rather than results from analyzing the data.

"Maximum Strategic Intransigence, Maximal Tactical Flexibility" (with Thanks to S. Reicher)

Our original stated aim was to investigate nonpathological cognitive ageing. We've stuck to that. Notwithstanding that continued focus, it soon became clear that we had useful data with regard to other aspects of healthy ageing and we investigated those—though never as a mainstream of the work. Also, as the participants grew older, some of them developed dementia, and we began to ascertain that and to use the information sometimes as an exclusion criterion and sometimes as an outcome (80).

Sometimes One Finds Something That is Too Good not to Develop

Indeed, that's what happened when we discovered that the SMSs' data were extant. Both Lawrence Whalley and Ian Deary were busy doing research but the opportunities seemed too important not to develop, that is, the possibility to study lifetime cognitive ageing with a childhood baseline cognitive test, and the chance to conduct linkage studies and find out whether childhood cognitive ability was related to survival (and, if so, why). Change of strategy? And the good luck of finding the SMSs' data was followed by the discovery of other data and the decisions about whether time spent in pursuing those was worthwhile. For example, we discovered birth records (including birth weight) of some of the people born in Edinburgh in 1921 (81). And we found out that some of the SMS1947 participants had had more information collected from them at age 11 and some into their 20s. We followed up both of these with add-on studies of, for example, cognitive ageing, cognitive epidemiology, personality, and life-long wellbeing (82–85). And we also found out that the Scottish "Midspan" studies had had many people born in 1921 and we obtained permission to link them to the Scottish Mental Survey 1932. This resulted in several contributions to our cognitive epidemiology work (86–90) and to our work on social mobility (91).

Only Set Up a Cohort if it has Something That it Can do That Others Cannot

Leading a cohort takes over one's life. The LBCs never had guaranteed funding beyond a 3- or 5-year grant-funding period. Yet, they have been funded continuously since January 1999. This means that the Director and co-investigators go home each evening with the responsibility to retain the cohort and the research team. Therefore, especially with the ease of accessing and analyzing secondary data from existing cohorts (with UK Biobank providing a current apogee for some investigations), one must ask why one is taking the trouble to set up a cohort, which will then put other people to the trouble of taking part. The why is that the cohort should be able to address an important set of scientific questions in a way that is valuable, that is, by being the only sample that can address the questions or at least by adding usefully to what can already be done in other samples. When we began the ABCs and LBCs, we knew of no other cohorts that could adjust for such a well-validated cognitive test in youth. About the best we were aware of was the cognitive surrogates employed in the Nun Study, which we found impressive.

Make the Cohort a Hub That Concentrates the Team's Scientific Expertise and Then Attach High-quality Spokes to Enhance the Cohort's Scope

The LBCs began with four people involved in the hub that designed, ran, and analyzed the LBC1921 study: Ian Deary (trained in medicine and psychiatry and a PhD in differential psychology), John Starr (geriatric physician), Martha Whiteman (PhD in psychology), and Alison Pattie (nurse and research assistant). Thus, the hub had expertise in cognitive testing, multivariate statistics (including structural equation modelling), other aspects of psychological differences, gerontology, and geriatrics. With time, the hub/core team enlarged—especially with the beginning of the LBC1936, at which time we added full-time individuals to look after the growing databases. Even early on though, we realized that we needed additional expertise, some of whom came from our Department of Psychology, some of whom were also from our University of Edinburgh, and some of whom were from other UK and overseas universities. Let's call these experts and their teams spokes to our hubs. (We shall see below that some spokes become part of the hub [yes, there, the metaphor breaks down a bit]). What spoke expertise did we add?: we brought in experts in molecular genetics, statistical genetics, brain imaging, ophthalmology, biomarkers, medical database linkage, telomere biology, neuropsychology, epidemiology, education, environmental geography, music, qualitative methods, physical activity, stem cell biology, postmortem pathology, molecular neuroscience, psychiatry, hematology, epigenetics, immunology, transcriptomics, lipidomics, proteomics… There are probably more and more will come, and we apologize to any whom we have forgotten to list. Oh, and we've tended to work with very good experts. It would be invidious to pick out a few, so the reader can spot them as co-authors on our articles. Finally, working with superb experts also means that some of that know-how in a new field rubs off on you—not a huge amount and we would never claim to be experts in our non-native fields—but enough that you are in a better position to spot new opportunities to occasionally contribute to new discoveries or perspectives in unexpected fields.

Consider Whether to Add an Expertise to the Core Team or to Outsource it to a Spoke

Looking at the LBCs' hub/core team as it is now, that is, those located in the same place in Psychology in 7 George Square at the University of Edinburgh, there are in-house geneticists and brain-imaging experts, for example, who would previously have been in spokes. Earlier on, a sole geneticist or brain imager would not have had an environment that would have nourished their expertise. Therefore, it was as we were able to attract more of such experts that we had a community that could help each other. Now, with >1 numbers of psychologists, brain imagers, geneticists (all of whom are also expert in multivariate analyses) in one place they can not only help their colleagues in the same field, they can conduct cross-disciplinary studies easily because they are colocated.

For Each Proposed Additional Variable, Ask Whether it is of Use in This Cohort

Generally, when we have decided to conduct an analysis or measure a variable in the LBCs we have asked ourselves these related questions: does the LBC make a valuable contribution to this literature?; and could this be done in any cohort or sample? In summary, we have tried to play to the strengths of the LBCs' information which means, a lot of the time, having childhood intelligence test scores in older people. However, as the LBCs' databases grow, other valuable opportunities emerge. For example, having longitudinal data on clonal hematopoiesis of indeterminate potential (92, 93) and DNA methylation, their linkage with existing LBC data was highly valuable and hard to replicate elsewhere. So, the more data that have been collected, the more opportunities there are for possibly-unique/at-least-valuable collection of still more (to some extent, and within what is practical and is sometimes driven by opportunity and/or serendipity) because there are so many data already collected to which they might be tested for association. Also worth saying is that the value in collecting new data during the later waves as sample sizes sadly dwindle also means that power is affected and there is less reason to add new data; to counterbalance that, there are lots of innovative ways in which one can continue to collect new data on the full sample by capitalizing on emerging methods and possibilities—for example, retrospective geocoding based on linked lifetime addresses in the full sample (e.g., ref. 94), medical record data linkage, analyzing blood samples stored from prior waves, and so forth. Thus, people become more rather than less interesting as they grow older.

Cover the Bases When Testing the Cohort

From our interest in contributors to nonpathological cognitive ageing, we knew those variables could come from a wide range of domains. Therefore, we had to gather a wide range of data. Of course, we looked around at other cohorts for guidance. We needed data from cognitive functioning, other aspects of psychology, social and demographic factors, lifestyle and health behaviors, demographics, biological and genetic factors, and medical information and fitness. These can be seen in our LBCs' study protocols and profile articles (4–7). One is inhibited from collecting what seems like too much, wishing not to fatigue the participants or discouraging them from returning. Our lesson was than older people can and will do more than you think. Initially, we were cautious with LBC1921 and more detailed and wide-ranging with the LBC1936 (who started with us when they were younger).

Even if They are not Perfect, Retain the Same Variables in the Next Wave of Testing

In psychology and in medicine and beyond, the ways of measuring things do not stay still. One chooses, say, cognitive and personality and mood and fitness tests (to pluck out a few from many types of data) for the baseline study. Sometimes, a newer and seemingly better test will appear after one has collected the data. What should one do? Hold your nerve: unless there is a big problem with the original test, collect the same thing again. There is value in longitudinal data with the same measures. Of course, if we have had time, we have included the better measure and the older measure at the next wave, but we have tended not to drop variables and we have been glad of that when it comes to longitudinal analyses.

If You Have Only 2 Min to Test a Person in a Cognitive Ageing Study, do the Wechsler Digit Symbol Test (Other, Equivalent Tests are Available)

Scores on this test age badly, that is, it declines more steeply than other cognitive tests and domains, and so it provides what one is looking for in a cognitive ageing study. Individual differences in the ageing of its cognitive domain—processing speed—correlate strongly with the individual differences in the ageing of other cognitive domains such as reasoning and memory.

Add the National Adult Reading Test if you have another two minutes (other, equivalent tests are available). This test will give you a decent estimate of the persons' peak prior cognitive ability, even when they have mild cognitive impairment or early dementia (82, 95). To an extent, it will

make up for not having the early life cognitive test scores that the ABCs and LBCs have, though it took these studies to establish that.

Add grip strength if you have another 2 min. It is a handy (pun intended) index of fitness and, in large samples, is predictive or mortality (tighter grippers live longer) (96, 97).

Do What You Can with Sufficient Power on Your Own and Join a Bigger Gang When You Can't

We have done under-powered studies. Many people have. We try not to. We set up the LBC1936, especially, with $N > 1000$, to be powerful in phenotypic studies looking at determinants of cognitive ageing. With over 700 of the LBC1936 having brain imaging data, it was one of the larger single-cohort studies of cognitive ageing with such data at the time. For *APOE* genetic studies, it was adequate in power. However, as soon as we began doing GWAS, with one exception (see below) we knew we had too little power to do anything that would be robust and so we collaborated. First, we had a UK-based gang that included us, the ABCs and the Manchester-Newcastle studies (22). It soon became clear that that was too small and we joined the CHARGE Neurology consortium, which had a cognitive group. That took the Ns to five and then six figures and only then did it seem that replicable results were appearing (24). Another example is the debate about replicable brain-behavior associations requiring thousands of samples (98). We also joined ENIGMA and other consortia because we recognize a similar situation in the brain imaging domain, though of course there remain things that can be done in LBC that aren't easy to find appropriate datasets for replication. By adopting multicohort approaches in the brain-imaging analyses, we lead we have shown that, actually, the LBC1936 solo results don't stack up too badly (45).

Having a Cohort That is Successful at Some Things Makes One all the More Appreciative of Other Cohorts That Can do Some Things Better

There are lots of studies out there with their different strengths. In the field of cognitive ageing, where we could pick out many good studies, we have a strong "we're not worthy" response to the ROSMAP studies which have an astonishing range of varied and good variables, a wonderful retention rate, and a terrific sign-up rate for postmortem donation. We have a similar response to UK Biobank, which is why we have spent so much time analyzing their data on topics relevant to the LBCs. The vision to collect such a large *N* (500,000)—with a very bold aim to collect with brain imaging data from 100,000 of them—means that the UK Biobank data is used world-wide.

Feel Free to Moonlight with Other Cohorts to Test Hypotheses That You Care About

Bearing in mind that we are interested in variation in brain and cognitive variables and their ageing, we have felt the need to be unfaithful to the LBCs when we can answer questions more powerfully elsewhere. The list is too long to name them all, but we have analyzed data and published results from UK Biobank, Generation Scotland, NLSY1979, The three British birth cohorts (1946, 1958, 1970), the West of Scotland Twenty-07 Study, and others.

Your Sample Can be a Control Sample for the Illnesses They Don't Have

Members of the Lothian Birth Cohort have been proud to appear as healthy controls in various medical studies. To pick out just two examples, they have been controls in genetic studies of colorectal cancer (99) and motor neuron disease (100).

You Will Regret the Things You Did Not Test

One can't go back and test at baseline again. One has to live with the decisions that were made. The LBC1936 will never have baseline brain imaging data at age 70, though they do have those data at every wave after that. So, think carefully about that initial testing wave. Related to that, you will bemoan, often, what the cohort does not have and perhaps envy other cohorts for having those data. For example, there are no contemporaneously-collected data in the LBCs between age 11 and older age (though we managed to find—via linkage to the Scottish Midspan study—data from middle age in some participants of the Scottish Mental Survey 1922 in our cognitive epidemiology work (86–90, 101)). It would have been helpful to have more early- and mid-adult variables collected in the LBCs. We have done our best to fill these gaps by retrospective self-reports and other techniques such as geo-linkages for past home addresses. One practical example from LBC1936 is that we did not ask for linkage to participant's medical records at the first wave of their testing at age 70; we corrected that, but we kicked ourselves for having omitted to request that from participants from the beginning.

Consider What Size of Cohort and Team is Optimal for Purposes (to Retain Focus) and Quality of Life (Though the Cohort will Take Over Your Life)

We have kept the LBC team to a moderate size. If one counts the PIs, the team that runs the study, the employed and ad-hominem/feminam postdoctoral fellows and research assistants—that is, mostly keeping it to the hub—the numbers vary around a score. All members of the team are encouraged to contribute to analysis and write-ups. We have no purely clerical staff—all are trained in science, usually psychology and/or genetics and/or brain imaging. Most are located in the same corridor or nearby.

If Something is Exciting, do it, Even if it is not in Your Field

We gave the example above of finding that DNA-methylation age was related to longevity. It was too exciting not to do. Even less related to our core mission than that was at the time when we had recently obtained the genome-wide scanning of SNPs in the LBCs. As an exercise for our newishly-appointed statistical molecular geneticist, we ran some biomarker variables through a GWAS procedure. We found three SNPs that accounted for 18% of the variance in activated partial thromboplastin time, and important measure in hematology (102). There was then the search for who had reported this already. No one had. We ran with it, with added hematological expertise. It was an interesting result and it was a good exercise in the analyzing and writing-up of GWAS. Perhaps even further from this was our involvement with clonal hematopoiesis (92, 93) which, again, seemed both too exciting and important not to become involved with.

We Learned That There Are Multiple, Partly Overlapping-Camps of Cognitive Ageing Research

Let's call these individual-differences psychology, experimental psychology, and medically-oriented. The individual differences approach might be exemplified by, say Timothy Salthouse or Warner Schaie, using large community-dwelling cohorts to examine patterns of cognitive ageing in different cognitive domains and what they share. The sample is all one group. Sometimes these studies are cross-sectional and sometimes longitudinal and sometimes cross-sequential. The experimental psychology approach might be exemplified by, say Michael Rugg, and is more likely to use smaller, separate samples of older and younger individuals and compare them on cognitive test scores. There are then more medically-oriented studies that focus on mild cognitive impairment and dementia. Sometimes these are case-control studies and sometimes cohorts that are followed into and through cognitive impairments.

Much of our work with the LBCs has been done within the individual differences framework. In part, this can involve data reduction statistical techniques (principal components analysis, factor analysis, sometimes in a structural equation modelling framework)—the bread and butter of differential psychology methods. However, the necessity of doing multivariate longitudinal modelling taught us that, with the LBCs, we were in one of the more technical analytical fields of psychology and also that the measurement of and determination of differences in cognitive (and other) change was, to say the least, much-discussed and sometimes fraught. Our team became familiar with, for example, growth curve modeling (32, 41, 62, 103). If one wants to study cognitive change and its determinants, one has to be prepared to learn to drive some heavy and complicated statistical machinery.

Take Biological Samples, Even if They do not Have an Immediate Use

From early on in our work with the LBCs we stored blood, plasma, and serum. We knew we needed these for basic health biomarkers (e.g., blood chemistry, hematology, glycated hemoglobin, etc.) and for genetics. We knew that more biomarkers would appear and that some would need to

be measured longitudinally. It is hard to exaggerate now how useful these samples have been. An indication of their uses can be seen in our cohort profile articles.

Future-proof the Cohort in Their Afterlife

The data from the LBCs will be analyzed after they are no longer with us and, we hope, after we are long-gone, too. However, there is a more biological meaning to this lesson. Having drawn cells which can be transformed to stem cells that can then be differentiated to many cell types means that the LBC1936 participants have provided material that can be used, in vitro, to test hypotheses about neural and other cell ageing (104). Related to that, the postmortem brain tissue donated by some of the LBC1936 participants who have died has already been used to investigate the biology of cognitive ageing, though the numbers to date are still very small (cf. the ROSMAP studies) (47, 105). Such discussion of attempted future-proofing of the LBCs reminds us to mention that this—and, indeed, the panoply of the LBCs—takes place within the strictures of consent and ethics and that these, during our work, have been moving targets. The consent required for different aspects of the studies has become more detailed as waves have passed, and postmortem brain tissue collection and storage and stem-cell creation and storage have each needed their own detailed consent procedures and ethical approvals. Other aspects of the study, such as linkage to medical records required additional (additional, i.e., to the ethical/consent work for the collection of the data within each wave) ethical/consent applications. From the beginning of the LBCs until the present we have proceeded via the ethical committees of the national Health Service in Scotland; that is, we have taken a medical rather than a psychological route to ethical approval and consent, which reflects the broad and health-related content of the studies.

Realize the Responsibility of Assembling a Longitudinal Cohort and Involve Them

One lives with a cohort. They are not like a convenience sample that one will thank and never see thereafter. One must form relationships with the cohort. One must listen to them and make channels for that to happen. With the LBCs, we have: newsletters (read some of them here: https://edin.ac/4dN8unc) at the ends of waves and at Christmas and at some other notable times; reunions at the ends of waves and at notable anniversaries (there are talks on the new results and question and answer sessions and information about the future plans; see our 20-year anniversary booklet here: https://edin.ac/3VnQiLi); and the LBCs' participants have made many national television, radio and newspaper appearances. There have been historical and art exhibitions (portraits some of the LBCs' participants and the research team) about the LBCs. There was a play about the LBCs performed at the Edinburgh International Fringe Festival. A film was made about the LBCs. A book was written recounting the personal histories of some of the participants and some of the team. The LBCs' participants have featured in umpteen science festivals, and knowledge exchange events for schoolchildren and members of the public. There's a summary here: https://edin.ac/4dTXQee. LBCs' participants and team members have twice been to the UK House of Lords to describe what their findings were to expert groups. When we wrote, above, that one must listen to the cohorts' members, that was not empty virtue signaling. Here's an example. At one of the reunions of the LBC1936, a participant asked why, given that we had collected so much information on them, we had not asked for their brain after death. That began a long process of obtaining permission for and setting up the LBC1936 Brain Tissue Bank (which has multiple small samples from brains, and not whole brains).

Being in an Observational Study Can be an Unintended Treatment

This probably has not happened often or to any large extent. However, being a participant in the LBCs, it would be impossible not to be alerted to aspects of cognitive and brain and more general ageing. To give just one example, one LBC1936 participant enrolled for and successfully completed a degree in philosophy with the UK's Open University because she thought she should use her brain more.

Referees and Journal Editors Want Longitudinal Data in Cognitive and Brain Ageing, but Funders Don't Want to Fund Them (Unless You Have New Hypotheses at Each Wave)

As we said above, the LBCs have never had guaranteed funding. However, we have had, for example, consecutive grants from Age UK that spanned the years between 2004 and 2020 for the LBC1936. We have also had multiple grants from the UK Research and Innovation bodies, especially BBSRC and MRC. Here, we are referring mostly to grants for core aspects of the study; there are many other grants for specific projects and for fellowships. But, as we note in parentheses above, it has never been sufficient to state, when applying for funding, that we were collecting another valuable wave of data from the LBCs. Almost always, we have had to develop fresh hypotheses for each wave. Although we have been able successfully to do this, and keep the show on the road (and deliver the specified work), the process of focusing on some specific hypotheses—from cohorts that have a solid track record of being a rich substrate on which to test so many hypotheses—was not easy.

Have a Good Succession, Even if it Happens During a Pandemic

Our cohorts have lasted a long time, beyond the full-time career of Ian Deary, for example. We were able to keep the LBC1936 cohort and research team going through the Covid-19 pandemic (106–108) and they are now (the second half of 2024) passing through Wave 7 at age about 88. The Deary-to-Cox succession is built not just on that one positive working relationship, but also upon great loyalty and continuity of team members and participants and collaborators. We still have, working in the team, Alison Pattie, who was first employed at the start of the LBC1921 in 1999, and Janie Corley, who was first employed at the start of the LBC1936 in 2004. Directing a cohort study is an intricate business, and we both count ourselves lucky to have benefitted from a superb and dedicated team, without whose continuity the whole operation would have been impossible to keep on the road.

Appreciate One's History

The history of the SMSs and the research environment in Scotland and nationally and internationally that brought them about has been a source of interesting study in itself. Central to that was the interesting figure of Professor Sir Godfrey Thomson, a giant in education, intelligence, and statistics and who is unfairly relatively unknown (109–113). His portrait hangs in the current director's office (along with an appreciation of *in umeris gigantum stamus*) as it did in the founding director's. Here is a link to a video covering the exhibition devoted to Thomson that we produced in 2016: https://www.youtube.com/watch?v=ZObidTDX4lI

And, now that the LBCs are 25 years old, they fold into and become part of that history. The next few years will see the age-90/Wave 8 testing of the LBC1936. Allied with that will be an effort to take our very large, securely-stored paper records and digitize them, so that every mark made for every test for every participant at every wave can be made available to future researchers. Also, the present authors have (it's a mug's game, though) tried to predict what might happen, scientifically, in the brain and cognitive ageing field over the next while (74).

Enjoy What You do

The LBC cohorts, the LBC research team, our collaborators, our funders (here, we make a special mention of Age UK, with whom we had a long and rewarding relationship), and the University of Edinburgh (with a special mention for the School of Philosophy, Psychology and Language Sciences and its various Heads for their support) are and have been enjoyable to work with. We value and humbly appreciate personal, social, and scientific premiums that return from that positive environment. The LBCs have opened doors that other types of research involvement wouldn't have: we and they met several members of the British Royal Family (including the late Queen Elizabeth II), Lords and MPs, and stars of stage and screen. Fuelled by the LBCs, we have seen junior researchers rise to professorships and to other valued vocational destinations; the LBCs have seeded cognitive and brain ageing researchers in other places.

Concluding Thoughts

The research with the Lothian Birth Cohorts was often summarized as something like, "to discover secrets of healthy cognitive ageing." In

various ways that handy-but-crude statement dissolved: first, we were sometimes confirming/incrementing others' findings rather than discovering (i.e., for the first time); second, we accepted that we should have to study pathological as well as healthy cognitive ageing, as the participants experienced dementia in larger numbers; and we expanded our outcomes remit beyond cognitive functioning. Those belt-loosening changes to our original methodological purity notwithstanding, we hope that discoveries from the LBCs, as enumerated in the "Scientific Discovery Lessons" will be useful to scientists in cognate fields. We also hope that our discoveries and incremental contributions to the fields in which we work will help people to make better choices regarding healthy lifestyles and provide understanding regarding contributions to individual differences in cognitive and brain ageing and ageing more broadly (alongside other teams' findings); we hope that scientists and lay people will appreciate that what they think are outcomes can be exposures and vice versa (reverse causation/confounding); this was all summarized in our "marginal gains" approach (8). We also refer the reader to our various policy-influencing attempts and contributions (here https://edin.ac/4dTXQee and here https://edin.ac/48te7G9) and also mention that we have undertaken hundreds of media and in-person appearances/activities to spread the word about good science and healthy ageing. These activities cover all ages from primary schools to older-people's groups and use educational programs, games, and art. Finally, we hope to have encouraged readers to find and read more of our publications and to keep up with those that appear in the coming years; they are listed here: https://edin.ac/3UixD26.

Data Availability Statement

No original data were generated in this work that requires public dissemination. Information about data access and collaboration for the Lothian Birth Cohorts of 1921 and 1936, the LBCs' data dictionaries, the LBCs' data summary tables, the LBCs' cohort profile articles, the LBCs' data request form, and data request contact information are all available here: https://edin.ac/3YkR3Ev.

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Author Contributions

Both authors contributed equally to all aspects of this work.

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Author Disclosures

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Prepartum bumetanide treatment reverses altered neonatal social communication but nonspecifically reduces postpubertal social behavior in a mouse model of fragile X syndrome

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Fragile X syndrome is caused by monogenic silencing of the *FMR1* gene and is characterized by high rates of autism spectrum disorder. A previous study demonstrated that prepartum administration of bumetanide, a chloride transporter blocker, normalized neonatal vocalization in non-congenic *Fmr1* knockout (KO) pups. However, the genuine contribution of *Fmr1* deletion to this phenotype in a congenic *Fmr1* KO mouse model and the long-lasting effect of prepartum bumetanide administration on postpubertal social interaction remains unclear. The current study aimed to determine the impact of prepartum bumetanide administration on vocalization at postnatal day 7 and social interaction at 6 and 8 weeks of age in a congenic *Fmr1* KO mouse model in which the genetic backgrounds were homogeneous between KO and wild-type (WT) littermates. Moreover, we applied a computational analytical algorithm and determined predictive variables of neonatal vocalization for postpubertal social interaction. Our data showed that (1) KO mice exhibited altered numbers and sequences of distinct call types during neonatal vocalization and reduced social interaction at 6 weeks, (2) select sets of neonatal vocalization variables predicted postpubertal social interaction levels, and (3) bumetanide restored neonatal vocalization in KO pups but nonspecifically reduced social interaction in WT and KO mice at 6 weeks. These data indicate that *Fmr1* deletion selectively impacts distinct elements of neonatal vocalization and postpubertal social interaction. Additionally, bumetanide selectively restores neonatal vocalization but has a transient nonspecific negative impact on subsequent postpubertal social interaction.

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Introduction

Precision medicine has not been utilized in psychiatry because the precise mechanistic targets of psychiatric disorders are not well established. Gene and genomic variants provide a reliable entry point for a mechanistic understanding of psychiatric disorders and mechanism-based therapeutic options (1).

Fragile X syndrome is a neurodevelopmental disorder caused by monogenic mutation and transcriptional silencing of the fragile X messenger ribonucleoprotein 1 (*FMR1*) gene, resulting in loss of its protein product, fragile X messenger ribonucleoprotein (FMRP). The syndrome includes the clinical diagnoses of autism spectrum disorder (ASD) and intellectual disability. Although *FMR1* silencing begins in the embryonic period in humans (2), the phenotypes are not reliably identified until later in the postnatal period, partly because clinical diagnoses are not feasible until formal tests can be reliably applied. However, many social, cognitive, affective, motor, and sensory phenotypes appear during early postnatal periods (3).

In the mouse brain, FMRP expression is high on embryonic day 11.5 (E11.5), E18.5, and during the first postnatal week but steadily declines thereafter (4, 5). The *Fmr1* peaks during the perinatal period mirror those in human brains (5). The developmental phase from the embryonic period to the first neonatal week, except for the time of birth (i.e., postnatal day 0, P0), is a unique period in which the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) exerts an excitatory action on neurons because of their high intracellular chloride concentration (6, 7). Remarkably, a seminal study by Ben-Ari and colleagues reported that this transient inhibitory role of GABA at and near P0 is attenuated in a mouse model of fragile X syndrome; when pregnant mothers were treated with bumetanide, an NKCC1 chloride transporter inhibitor, one day before delivery, the increased probabilities of two neonatal call types (i.e., chevron and downward) were normalized in non-congenic *Fmr1* knockout (KO) pups at P8 (8). Human studies have not addressed the critical period for bumetanide treatment due to technical and ethical issues. The therapeutic effects of bumetanide on patients with idiopathic ASD are largely negative when treatment starts at 2 years of age or later (9).

The impetus of the present study was 3-fold. First, a non-congenic mouse model poses an interpretative issue. Although non-congenic mouse models have randomly shuffled allelic distributions throughout the genomes of KO and wild-type (WT) littermates, they have a systematic, consistent bias near the targeted gene. Because of the low recombination rates between the targeted gene and genes located nearby, the alleles of nearby genes of the targeted gene tend to be inherited together. Because gene targeting is induced in embryonic stem (ES) cells of the 129/Sv substrains and mice are bred with another strain (e.g., FVB or C57BL/6J), KO offspring accumulate alleles of neighboring genes derived from ES cells, and WT offspring accumulate alleles of breeders. Because these strains differ in their molecular, cellular, electrophysiological, anatomical, and behavioral phenotypes, any phenotypic differences between non-congenic KO and WT littermates cannot be unequivocally attributed to the targeted gene (10). We addressed this issue using a congenic *Fmr1* KO mouse model.

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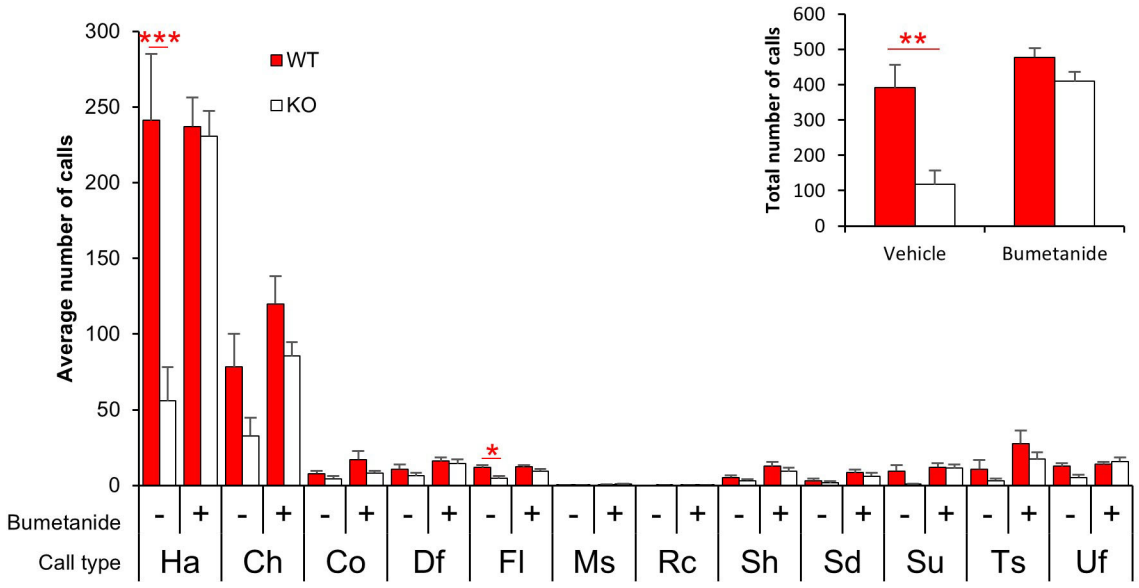

Figure 1. Number of neonatal vocalization call types. The average number (±SEM) of each call type emitted by male WT and KO mice is shown. Asterisks (*, **, and ***) indicate statistically significant differences (1%, 0.5%, and 0.1%, respectively) between WT and KO pups. Inset: WT and KO mice differed ($F(1,41) = 18.839$, $p < 0.0001$) and bumetanide increased calls in KO mice (Treatment, $F(1,41) = 23.080$, $p < 0.0001$; Interaction, $F(1,41) = 6.7576$, $p = 0.0129$). Student *t*-tests showed that WT and KO mice differed in the total number of all calls without bumetanide treatment ($p = 0.0013$), but not with bumetanide ($p = 0.0850$). Main figure: WT and KO mice differed depending on call types and treatment (Genotype x Call types x Treatment, $F(11,451) = 8.8573$, $p < 0.0001$). Vehicle-treated WT and KO pups differed in the numbers of harmonic ($p = 0.0007$) and flat ($p = 0.0072$). These significant differences of *t*-tests survived Benjamini–Hochberg's corrections for multiple comparisons at 5% FDR. Bumetanide-treated WT pups did not differ in any call type from bumetanide-treated KO pups ($p > 0.05$). Vehicle: WT, $n = 9$; KO, $n = 12$. Bumetanide: WT, $n = 12$; KO, $n = 12$. Ha, harmonic; Ch, chevron; Co, complex; Df, down-frequency modulation; Fl, flat; Ms, multi-step; Rc, reverse chevron; Sh, short; Sd, step-down; Su, step-up; Ts, two-step; Uf, up-frequency modulation.

Second, phenotypic variability is a norm, and thus comparisons based on group averages do not fully capture the nuanced nature of the impacts of genomic variations. *Fmr1* mutation does not cause complete penetrance, and approximately 60% of male carriers are diagnosed with ASD. Elements of ASD also show phenotypic variability; social communication is weak among *Fmr1* mutation carriers with an ASD diagnosis (3). More than 10 distinct call types and their specific temporal sequences are uniquely impacted by dose alterations of genes implicated in neurodevelopmental and psychiatric disorders in mouse models (11–13). Therefore, to capture the variable nature of this syndrome and its dimensional elements, we applied computational approaches (11, 12, 14, 15).

Third, specific roles of GABA during the perinatal period in the development of postpubertal social interaction have not been explored. This issue is pertinent to the theoretical question of whether normal neonatal social communication is a prerequisite for normal social interaction at later times or whether these two processes independently develop. We addressed this question by computationally evaluating the predictive power of variables of neonatal vocalization for postpubertal social interaction without and with bumetanide. Differential alteration of phenotypes of the two developmental stages by this treatment would support mechanistically independent processes; in contrast, the presence of predictive neonatal variables for postpubertal social interaction and improvement of both behaviors by bumetanide at the two developmental stages would be consistent with the hypothesis that the two developmental stages share a common mechanistic basis.

To test the hypothesis that a GABAergic tone during the perinatal period is a determinant for neonatal social communication and postpubertal social interaction, we administered bumetanide prepartum and evaluated its impacts on neonatal vocalization at P7 and on postpubertal social interaction at 6 and 8 weeks of age. As fragile X syndrome is associated with variable expressivity and developmental trajectories among carriers, we applied a computational analytical algorithm (12) to predict the variability of postpubertal social interaction scores from the variation in neonatal vocalizations.

Results

Fmr1 Deletion Alters the Number of Calls with Specific Geometric Shapes

Evidence indicates that the perinatal/neonatal period is critical for the development of social behavior (16), and neonatal vocalization is the earliest expression of social communication in rodents and humans (11, 12, 17, 18). It was reported that the probabilities of calls with two specific geometric shapes, termed chevron and downward, were increased and these increases were normalized by bumetanide in a non-congenic mouse model of fragile X syndrome (8). However, mouse pups generally exhibit more than 10 distinct call types, and they are distinctly impacted by genes linked to neurodevelopmental disorders (11–13, 19, 20). In particular, the call types that are altered in various mouse models of fragile X syndrome vary depending on genetic backgrounds, sex, age, call classifications, and other factors. When neonatal call types were analyzed in mouse models of fragile X syndrome in which the genetic backgrounds were made homogenous between WT and KO pups, various call types were found to be altered, resulting in greater numbers of "frequency jump" calls, which included two-syllable and frequency step calls, at P7 (13), increased percentages of chevron and frequency step call types and decreased percentages of complex, composite, downward, harmonic, two-syllable, and short call types at P8 (21).

We comprehensively characterized call types that were altered by *Fmr1* deletion and bumetanide treatment in congenic *Fmr1* KO pups and their WT littermates. KO pups emitted fewer total calls than WT pups, and this effect was ameliorated by bumetanide at P7 (Figure 1 inset). The numbers of the 12 call types were separately compared between WT and KO mice (Figure 1; Supplemental Table S2). Compared with WT pups, KO pups emitted fewer harmonic and flat call types. Bumetanide increased the number of calls in KO pups to the extent that KO and WT pups did not differ for any call types.

We additionally examined the proportion of each call type within the total number for each pup. In this measure, WT and KO mice did not differ for call types and bumetanide had no significant effect (Supplemental Figure S1; Supplemental Table S2).

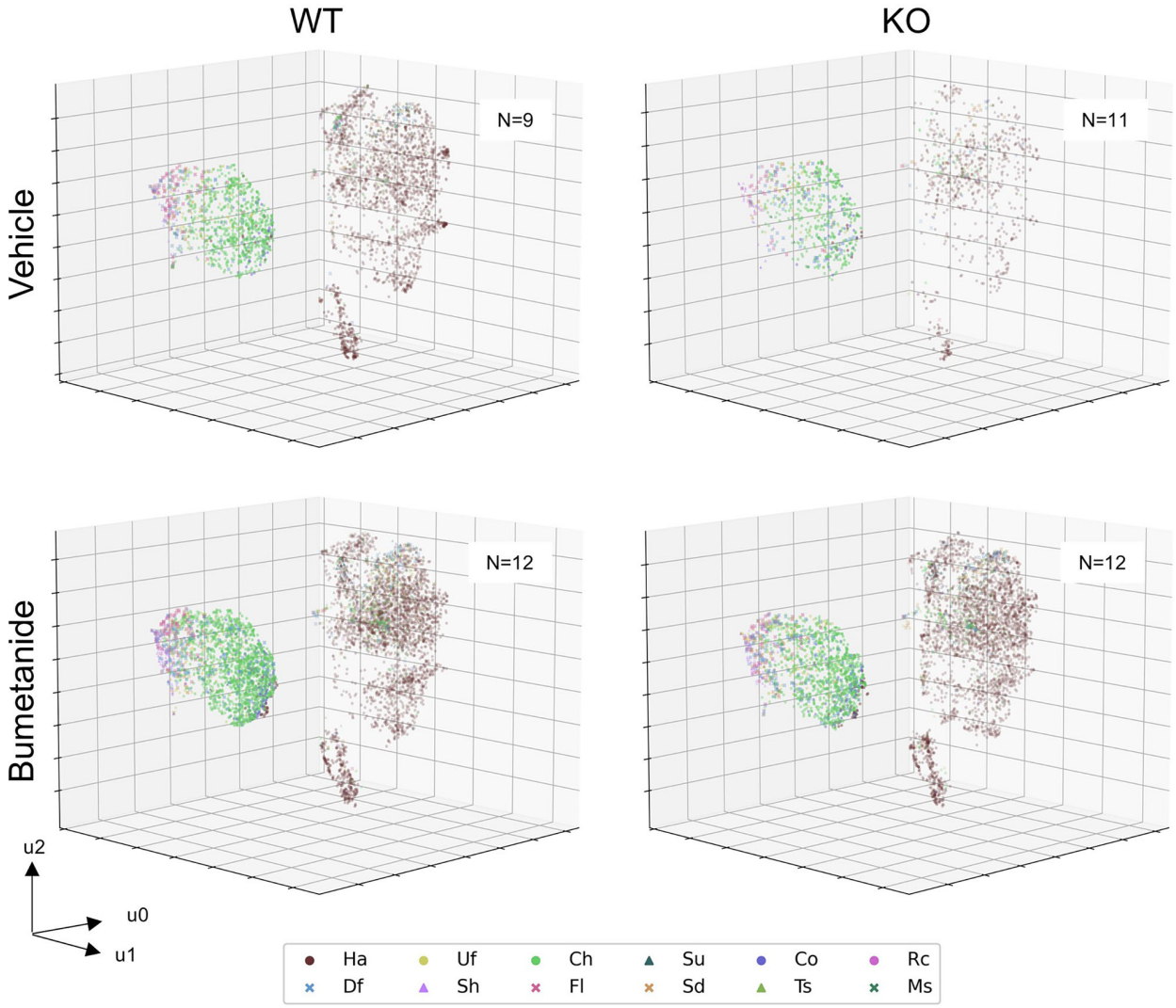

Figure 2. Three-dimensional UMAP of VocalMat parameters. UMAP was used to convert the quantitative acoustic parameters into lower dimensions while maintaining their character. The VocalMat parameters included "duration," "min_freq_main," "max_freq_main," "mean_freq_main," "bandwidth," "min_freq_total," "max_freq_total,""mean_freq_total," "min_intens_total," "max_intens_total," and "mean_intens_total". Calls were separated into three clusters. Call types are indicated by distinct colors in the three clusters.

The length of all calls (Supplemental Figure S2 inset) or each call type (Supplemental Figure S2) did not differ between WT and KO pups. While the lengths of all calls were prolonged by bumetanide (Figure 2 inset), they did not reach significance when each call was separately analyzed (Supplemental Figure S2).

Fmr1 Deletion does not Alter the Quantitative Acoustic Parameters of Call Types

These analyses were based on the categorical classification of geometric call shapes. However, each call type also has quantitative acoustic parameters. We thus additionally analyzed the (1) bandwidth in Hz, (2) maximum, mean, and minimum frequencies in Hz of a call or its main components, and (3) maximum, mean, and minimum intensities of each call. Vehicle-treated WT and KO pups did not differ in any of these acoustic parameters for all call types (Supplemental Table S3A–S3J). Thus, the acoustic parameters did not discriminate genotype.

Fmr1 Deletion Impacts the Quantitative Parameters of Calls in a Dimensional Space

Having established that the simple acoustic parameters did not differentiate genotype or drug treatment, we next aimed to evaluate calls in an independent dimension. We visualized and evaluated the complex nature of VocalMat's quantitative acoustic parameters in three and two dimensions using Uniform Manifold Approximation and Projection (UMAP).

We first used three commonly used UMAP metrics: Manhattan, Euclidean, and Chebyshev (Supplemental Figure S3). To evaluate the relationship between these UMAP clusters and call types, we color-coded each data point based on the call classification of our modified VocalMat. Because the Euclidean metrics provided better cluster separation than the Manhattan or Chebyshev metrics, we used the Euclidean metric for all the subsequent analyses.

This analysis reduced all calls into three main spatial clusters in a three-dimensional UMAP space (Figure 2; Supplemental Figures S4 and S5). The decreased numbers of calls are apparent in all three clusters in KO pups; all these spatial clusters were restored by bumetanide. Harmonic calls were predominantly represented in spatial Cluster 1 and Cluster 3 (Supplemental Figures S5 and S6); chevron was the most predominant call type in Cluster 2 (Supplemental Figures S5 and S6). This analysis showed quantitative differences and similarities among VocalMat-based call types. Calls classified as "harmonic" have the most variable quantitative profile than other call types.

The combined analytical approaches for categorical call types and quantitative acoustic parameters showed that *Fmr1* deletion and

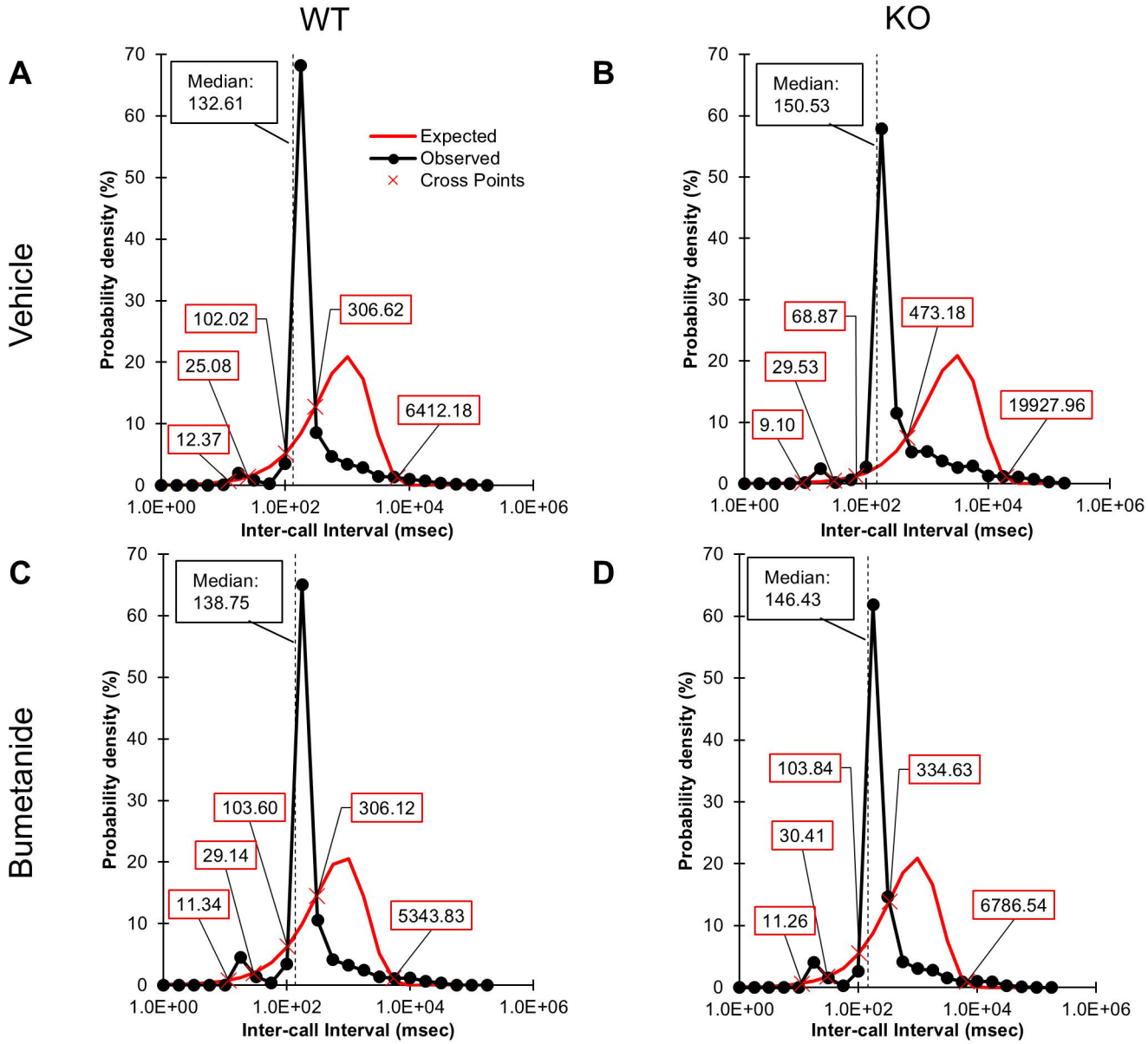

Figure 3. Probabilities of intercall intervals. The observed and expected probabilities of intercall intervals of vehicle-treated WT (**A**), vehicle-treated KO (**B**), bumetanide-treated WT (**C**), and bumetanide-treated KO mice (**D**). Vehicle-treated KO pups had longer intercall intervals, compared with vehicle-treated WT pups ($p < 0.0001$). Bumetanide reduced intercall intervals in KO pups ($p < 0.0001$) but bumetanide-treated KO pups still maintained longer intercall intervals than bumetanide-treated WT pups ($p < 0.0001$). The actual intercall intervals (ms) at cross-points of the observed and expected probabilities are shown. Intercall intervals shorter than the higher cross-points between the observed and expected distributions had probabilities higher than the expected distributions and thus are considered intervals between calls within sequences (Vehicle-WT, 306.62 ms; Vehicle-KO, 473.18 ms; Bumetanide-WT, 306.12 ms; Bumetanide-KO, 334.63 ms); intercall intervals longer than the cross-points were considered intervals from the end of a sequence to the beginning of the next sequence.

bumetanide impact calls with distinct categorical and quantitative parameters.

Fmr1 KO Alters the Temporal Distribution of Call Sequences

Calls were emitted with various intercall intervals. To objectively identify a temporal cluster of calls, we first determined the Poisson distribution, a theoretically expected distribution of intercall intervals with a given number of calls within a 300-s test time (Figure 3, red lines). Because KO pups emitted fewer calls than WT pups, the theoretically expected intercall intervals of KO pups were shifted to the right (Figure 3B, red lines) compared with those of WT pups (Figure 3A, red lines). These data indicate that *Fmr1* deletion alters the intercall intervals without changing the length of each call (see Supplemental Figure S2).

The largest peaks of observed intercall intervals, representing the most frequent intercall intervals, were found around 200 ms (Figure 3, black lines). There were also much smaller peaks that occurred more frequently than expected from the Poisson distributions below approximately 30 ms (Figure 3, black lines). However, these short intercall intervals occurred in less than 5% of all calls of all pups.

We defined a call sequence as a series of calls that were emitted with intercall intervals shorter than the larger value of the two crosses between the highest probability peak of the observed curve and the expected probability curve (see Figure 3A, WT, Vehicle, 306.62 ms; B, KO, Vehicle, 473.18 ms; C, WT, Bumetanide, 306.12 ms; D, KO, Bumetanide, 334.63 ms).

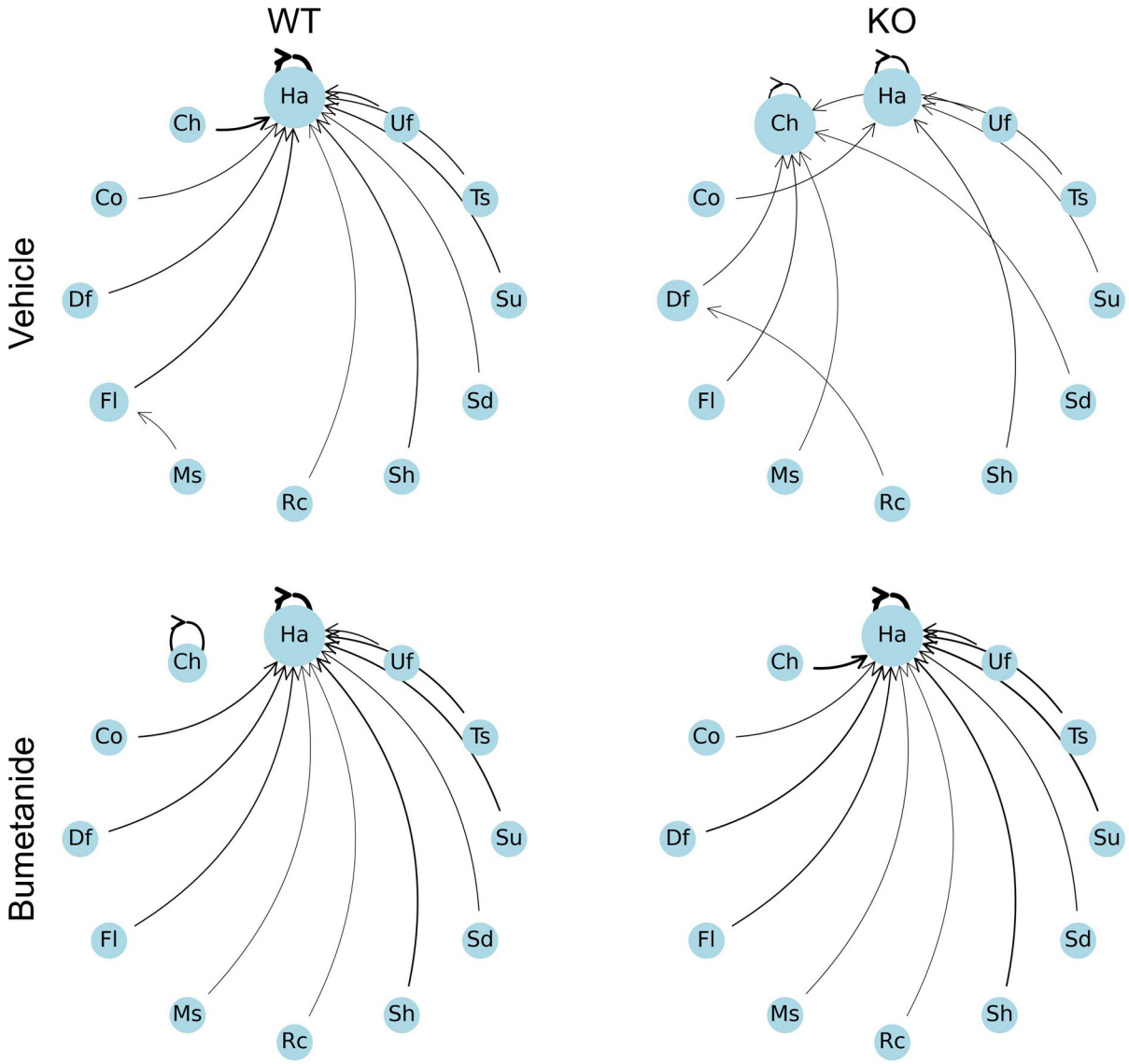

Figure 4. Markov model. The relative probabilities of each two-call sequence for each of the 12 call types identified by the Markov models are shown. The sum of all proportions of each starting call is 1, and the sum of all Markov probabilities is 100% × 12 calls. The line thickness represents the relative probability of a call sequence.

When call sequences were so defined, short and long sequences were identified around 100 ms and 1 s, respectively (Supplemental Figure S7). Bumetanide lengthened the long sequence duration in KO pups compared with that in vehicle-treated KO pups.

Fmr1 KO Alters Call Sequences

Having defined call sequences, we next determined how various call types were ordered within sequences. We determined the probabilities of two consecutive calls for a given starting call using Markov modeling. This model is based on the Markov property, where future states depend only on the current state. The probabilities of call sequences were determined based on two consecutive calls. In other words, the probability of the next call type was computed within each call type; the sum of probabilities of all two-call sequences starting from a given call type was always 1.0. Thus, this analysis was not influenced by the probabilities of the first call of two consecutive calls emitted by each subject. This analysis revealed distinct frequently emitted two consecutive calls of vehicle-treated WT and KO pups (Figure 4). Bumetanide restored the altered two-call sequences of KO pups.

Because of the Markov property, the two-call sequences starting from less emitted call types tend to be overestimated. To evaluate call sequences within each mouse with all starting call types, we incorporated a separate analysis of how frequently each two-call pair was emitted. In this analysis, the sum of probabilities of all two-call sequences starting from all call types was always 1.0. Two-call pairs starting and ending in harmonic were predominant in all groups (Supplemental Figure S8).

Prepartum Bumetanide Treatment Reduces Postpubertal Social Interaction in KO Mice

When the pups reached 6 and 8 weeks of age, they were sequentially tested for social interaction. We tested naturalistic social interaction in a home cage setting because it is a better validation than other procedures (22), and molecular mechanisms underlying direct social contact in such a set-up and indirect contact with a barrier differ (23).

There were a few test or stimulus partner mice in each group that exhibited aggressive behavior. Such cases were eliminated from the analysis (24) for three reasons. First, genetic bases of affiliative social interaction and aggressive behavior are nonidentical (25). Second, aggressive

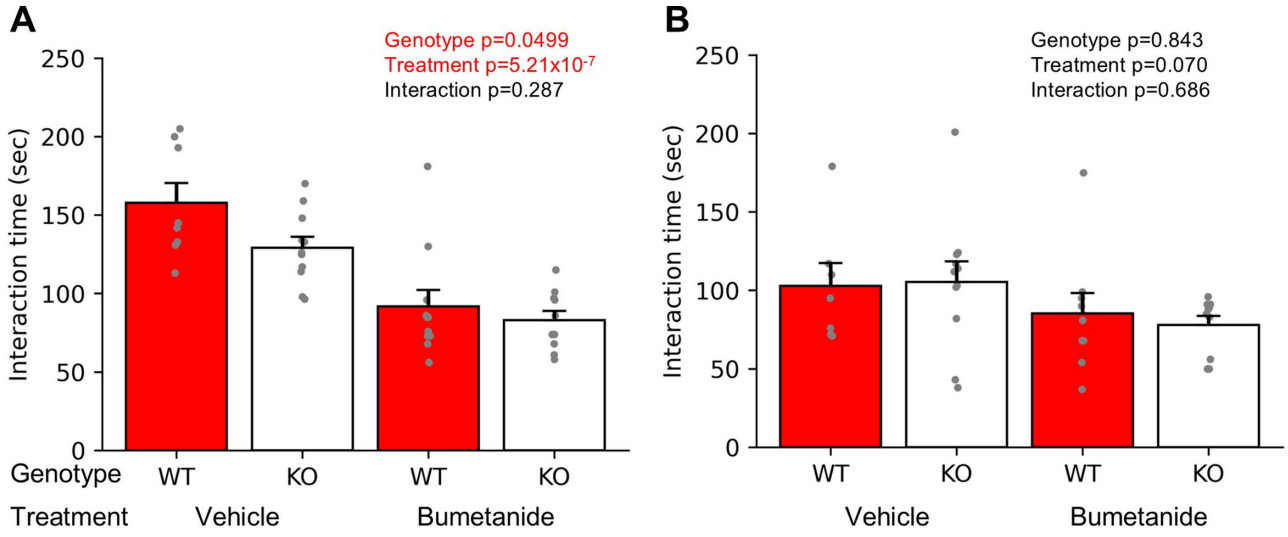

Figure 5. Social interaction. The time (mean±SEM) spent in active social interaction at 6 weeks of age (**A**) and 8 weeks of age (**B**) is shown. (**A**) KO mice spent less time in social interaction than WT mice (Genotype, $F(1,36) = 4.1160$, $p = 0.0499$) and bumetanide treatment equally reduced social interaction in both genotype groups (Treatment, $F(1,36) = 37.1341$, $p < 0.0001$; Interaction, $F(1,36) = 1.1674$, $p = 0.2871$). WT/Vehicle, $n = 8$; KO/Vehicle, $n = 11$; WT/Bumetanide, $n = 11$, KO/Bumetanide, $n = 10$. (**B**) WT and KO mice did not differ (Genotype, $F(1, 33) = 0.0399$, $p = 0.8429$) and the effect of bumetanide treatment was not significant ($F(1,33) = 3.4975$, $p = 0.0704$) without an interaction effect ($F(1,33) = 0.1661$, $p = 0.6862$). WT/Vehicle, $n = 7$; KO/Vehicle, $n = 11$; WT/Bumetanide, $n = 9$, KO/Bumetanide, $n = 10$.

behavior is not a prominent element of fragile X syndrome, but altered affiliative social interaction is an element. Third, aggressive behavior indirectly suppresses the occurrence of affiliative social interaction, thereby artificially underestimating it. The following test mice were eliminated in each group: 6 weeks: Vehicle WT, 1 mouse; Bumetanide WT, 1 mouse; Bumetanide KO, 2 mice. 8 weeks: Vehicle WT, 1 mouse; Bumetanide WT, 3 mice; Bumetanide KO, 1 mouse. We also eliminated cases where the stimulus mice were agitated and hyperactive (6 weeks, Vehicle KO, 1 case. 8 weeks: Vehicle WT, 1 case; Bumetanide KO, 1 case), as such behavior of the stimulus mouse makes it physically impossible for a test mouse to engage in affiliative social interaction.

KO mice showed lower social interaction levels than WT mice; prepartum bumetanide treatment equally lowered social interaction levels in both WT and KO mice at 6 weeks of age (Figure 5A).

At 8 weeks of age, vehicle-treated WT and KO mice did not differ, and bumetanide had no statistically significant effect on social interaction in WT and KO mice (Figure 5B).

Predictive Variables of Neonatal Vocalization for Postpubertal Affiliative Social Interaction

If a mechanistic link exists between neonatal vocalizations and the development of postpubertal social behavior, the former should predict the latter. To identify such predictive variables of neonatal vocalizations among the numbers and probabilities of call types and call sequences, we developed Least Absolute Shrinkage and Selection Operator (Lasso) regression models for each group. The acoustic variables were not included in this analysis, as there was no difference in their averages between genotypes or treatments, and some call types were not emitted in some mice, thus providing no acoustic parameter (e.g., bandwidth and amplitude) (Supplemental Table S3).

This analysis identified a unique set of specific variables within each group that predict affiliative social interaction levels at 6 weeks of age (Figure 6). The most robust predictors of vehicle-treated WT mice were the number of flat->chevron and the proportion of chevron->chevron; those of vehicle-treated KO mice were the Markov probability of chevron->short, and the non-Markov proportions of harmonic->chevron and chevron->two-step calls. These variables were also significantly correlated with the scores of social interaction (see Figure 6 inset; Supplemental Table S2; Supplemental Figure S6).

In bumetanide-treated mice, several variables of neonatal vocalization were identified as predictors by Lasso models (Figure 6). The most robust predictors were the proportion of two steps->flat and Markov probability of harmonic->step-up in WT mice; the Markov probabilities of step-down->harmonic, harmonic->chevron, complex->upward frequency modulation, and chevron->two steps, and non-Markov proportions of downward frequency modulation->flat in KO mice. Remarkably, none of the Lasso-selected variables were significantly correlated with social interaction scores in bumetanide-treated WT or KO mice (Figure 6 inset; Supplemental Table S2; Supplemental Figure S6). This result is likely due to the nonspecific effects of bumetanide on social interaction in both WT and KO mice (see Figure 5).

In the analysis above, we identified neonatal call parameters that best predicted postpubertal social interaction within 6-week vehicle-treated WT or KO mice. We next identified neonatal call parameters that best differentiated social interaction scores and genotypes in pooled data of both vehicle-treated WT and KO mice at 6 weeks. First, a Lasso regression model with social scores as the coefficient identified sequences and call numbers as predictors of social interaction (Supplemental Figure S9A, e.g., Fl->Ch, Sh->Fl). Only two of them were significantly correlated with social interaction scores (Fl->Ch and Ch->Sh; Supplemental Table S2; Supplemental Figure S9A). Second, a Lasso regression model identified the number of Flat->Chevron as a predictor of the genotype (Supplemental Figure S9B). However, this predictor failed to discriminate the genotype (see Supplemental Figure S9B and Supplemental Table S2).

Although there was no effect of genotype or bumetanide on social interaction at 8 weeks of age (Figure 5), we applied the Lasso method to determine which neonatal vocalization variables predict individual variation in social interaction (Supplemental Figure S10). Distinct sets of two-call sequences predicted individual variation within each genotype, including the Markov probability of complex->chevron and the non-Markov proportion of chevron->complex in vehicle-treated WT mice. In vehicle-treated KO mice, the Markov probability of upward frequency modulation->two steps and the non-Markov proportion of harmonic->chevron were robust predictors.

In bumetanide-treated WT mice, Lasso models identified some sequences (e.g., complex->upward frequency modulation and two-step->upward frequency modulation; Supplemental Figure S10), all of which

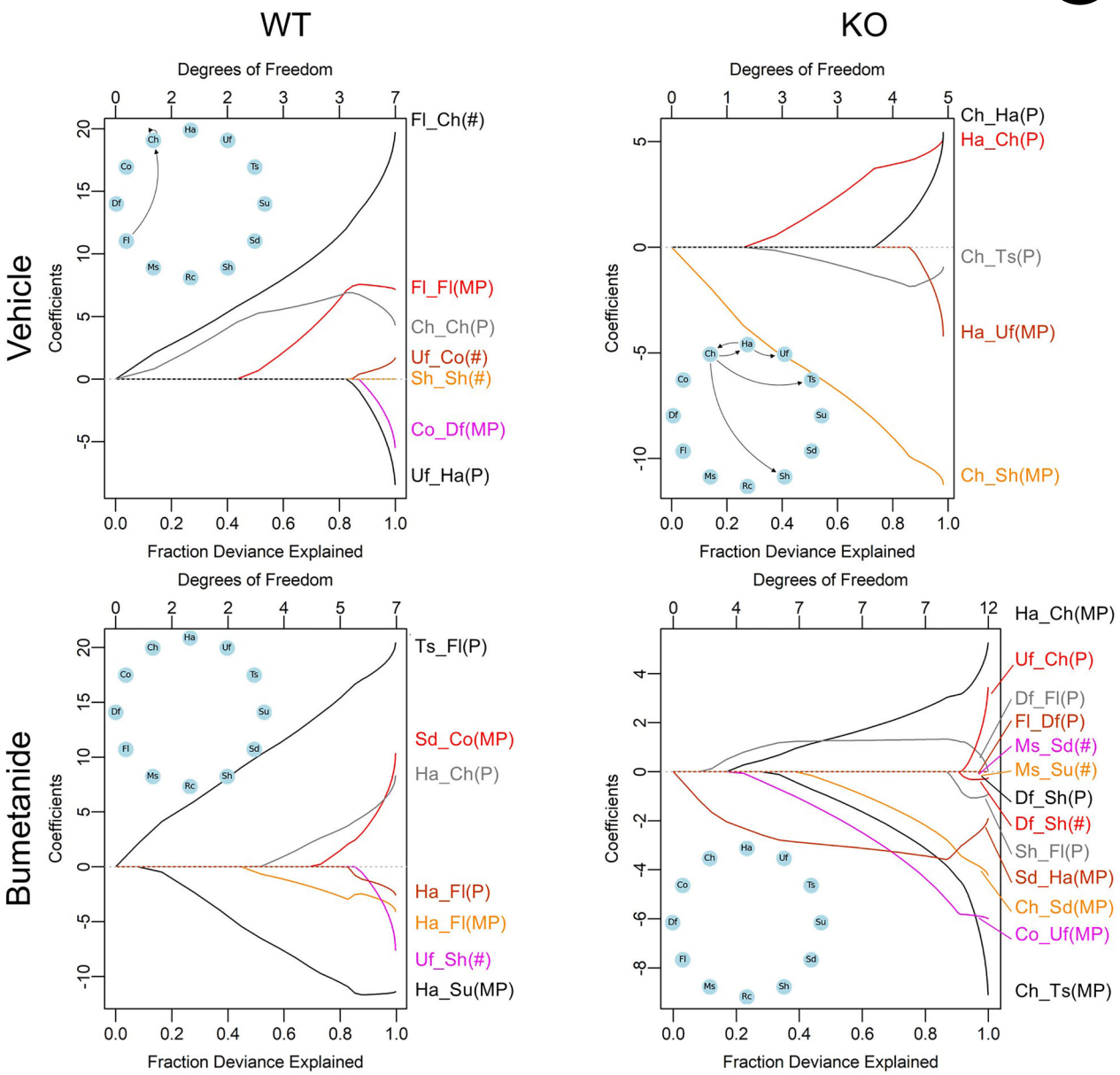

Figure 6. Neonatal vocalization predictors for social interaction at 6 weeks of age. The fraction of deviance explained and the coefficients of variables were determined by the Lasso regression model. All parameters (call type number and proportions, call sequence number, Markov probabilities, and non-Markov proportions) were used for selection. Lasso models identified several predictors for social interaction scores within each group. Each inset shows call sequences that were significantly correlated with the individual post-pubertal social interaction scores (see Supplemental Table S2 and Figure 6). The Markov probabilities, non-Markov proportions, and numbers of two-call sequences were selected. The small values along the fraction of deviance explained axis are robust predictors of social interaction. Ha, harmonic; Ch, chevron; Co, complex; Df, down-frequency modulation; Fl, flat; Ms, multi-step; Rc, reverse chevron; Sh, short; Sd, step-down; Su, step-up; Ts, two-step; Uf, up-frequency modulation, MP, Markov probability; P, non-Markov proportion; #, number.

were significantly correlated with the postpubertal social interaction scores (Supplemental Figure S10 inset; Supplemental Table S2).

In bumetanide-treated KO mice, a Lasso model selected the Markov probability of flat->upward frequency modulation and upward frequency modulation->upward frequency modulation and number of short->short as the most robust predictors for social interaction (Supplemental Figure S10), none of which were significantly correlated with the social scores (Supplemental Figure S10 inset; Supplemental Table S2).

Discussion

We hypothesized that the intracellular concentration of chloride ions in neurons around the time of birth is a critical determinant for the causal sequential development of neonatal and postpubertal social behaviors. This hypothesis was based on the pioneering report that bumetanide, an NKCC1 (Na^+-K^+-$2Cl^-$ cotransporter) inhibitor that controls intracellular chloride ion concentration, restored neonatal vocalizations in non-congenic *Fmr1* KO pups (8). We used a congenic *Fmr1* KO mouse to determine the predictive variables of neonatal vocalization for the post-pubertal social interaction scores as well as the impact of bumetanide on these developmental variables. Our data showed that (1) *Fmr1* deletion reduced the number of specific neonatal call types and probabilities of call sequences; (2) bumetanide restored these neonatal phenotypes; (3) *Fmr1* deletion reduced direct social interaction at 6 weeks, but not at 8 weeks, of age; (4) bumetanide nonspecifically reduced the postpubertal social interaction level in WT and KO mice, resulting in indistinguishable

social interaction levels at 6 weeks; (5) distinct neonatal vocalization call sequences predicted the postpubertal social interaction level in vehicle-treated WT and KO mice at 6 weeks; and (6) such predictors did not exist in bumetanide-treated WT and KO mice because bumetanide nonspecifically reduced social interaction scores to the level where the two genotype groups were indistinguishable.

Unless unavoidable, we will discuss only those studies that used congenic or coisogenic *Fmr1* KO mice lines or an F1 hybrid line where the homogeneous genetic backgrounds of WT and KO mice are maintained. Non-congenic mice with "mixed" genetic backgrounds consistently carry more breeder line alleles in WT mice and more ES cell alleles (e.g., 129/Sv) in KO mice near the deleted gene (10). Such models do not identify phenotypes that genuinely reflect the impact of *Fmr1* deletion alone. Moreover, we limit our discussion to studies that manually classified calls or used automatic call classification systems with low false negative and false positive rates because high false negative and positive rates compromise the accurate evaluation of neonatal calls (26).

We observed that male congenic *Fmr1* KO mice emitted fewer calls of specific call types (harmonic and flat) but exhibited normal percentages of all call types. Previous well-controlled studies identified alterations of various neonatal vocalization parameters in congenic *Fmr1* KO pups. Congenic *Fmr1* KO pups with an FVB background emitted more two-syllable and frequency steps at P7 than WT pups; however, sex was not specified in this study (13). In another study, male congenic *Fmr1* KO pups with an FVB background emitted fewer calls at P9 and P13 and more calls at P12 than WT pups (27). Our results are consistent with this observation in that KO pups emitted fewer calls than WT pups (see Figure 1 inset). While power analyses indicate that statistically significant differences might be achieved with larger sample sizes for chevron ($n = 22$), step-up ($N = 13$), up-frequency modulation ($N = 15$), all the other call types are estimated to require much large sample sizes (see Supplemental Table S2 and Figure 1, Power analysis). Thus, *Fmr1* KO affects harmonic and flat call types more robustly than other calls.

When calls were analyzed by proportions, we found decreased proportions of harmonic and two-step calls and higher proportions of chevron in KO pups. However, none of these alterations reached statistical significance. In a previous study, male *Fmr1* KO pups with an FVB background produced proportionally more chevron and frequency step call types and decreased proportions of complex, composite, downward, harmonic, two-syllable, and short call types at P8 (21). A larger sample size of their study (WT, $n = 17$; KO, $n = 13$) is one likely factor for significance in more call types in their study than in our study (WT, $n = 9$; KO, $n = 12$). Power analyses showed that with larger sample sizes, vehicle-treated WT and KO mice are likely to achieve a statistically significant reduction in proportions of harmonic ($N = 13$) and step-up (similar to two-syllable, $N = 45$) calls and increases in down-frequency modulation (similar to downward) ($N = 39$) and short ($N = 48$) calls (Supplemental Table S2; Supplemental Figure S1). The other call types require much larger sample sizes to achieve statistical significance: chevron ($N = 69$), frequency step (multiple steps, $N = 830$; two steps, $N = 704$), complex ($N = 638$) call types (Supplemental Table S2; Supplemental Figure S1). Together with these power analyses, our data clearly indicate that *Fmr1* deletion impacts the proportions of harmonic, down-frequency modulation, step-up, and short calls in this order.

A previous study demonstrated that non-congenic *Fmr1* KO mice exhibited *higher* probabilities of chevron and downward call types than WT pups at P8 (8). Our study showed that the percentages of chevron and down-frequency modulation calls were higher in male KO pups than in male WT pups at P7 (Supplemental Figure S1), and bumetanide tended to correct these trends when using sample sizes ($n = 9$–12) similar to those in the previous study (8) ($n = 9$–13). However, none of these trends achieved statistical significance in our study, although a power analysis indicates that an increased proportion of down-frequency modulation ($N = 39$) and chevron ($N = 69$) calls in KO mice may achieve significance with much larger sample sizes (see Supplemental Table S2 and Supplemental Figure S1). Several factors are likely. First, the study by Tyzio and colleagues eliminated pups that emitted less than 50 calls during a 3-min period, but we did not eliminate such cases because we considered low call numbers as a phenotype. Second, our and their recording time durations were 5 min and 3 min, respectively. The phenotype they detected might occur in the first 3 min of our testing. However, this was not the case; when data of the first 3 min were analyzed, we still did not find statistically significant increases in chevron or downward frequency modulation (Supplemental Table S2, Figure 1_Number_3 min; Supplemental Table S2, Figure 1_Proportion_3 min). Third, the study by Tyzio and colleagues did not determine the sex of mice used, whereas our study used males only. Fourth, their study analyzed and presented only chevron and downward call types. Despite these methodological differences, our data are consistent with their finding that *Fmr1* deletion alters the proportions of specific neonatal call types, and the effect is normalized by bumetanide. Our study further showed that bumetanide restored defective call sequences of KO pups to levels similar to those of WT pups. While a larger sample size might identify more call types that differ between the genotypes, it is clear that some call types (e.g., harmonic call types) are more easily affected by *Fmr1* deletion than others.

Our observations further extended the study of Tyzio and colleagues by including an analysis of postpubertal social interaction and the effects of bumetanide on this phenotype. *Fmr1* deletion impaired postpubertal direct social interaction at 6 but not 8 weeks of age. Previous studies of *Fmr1* KO mice did not consistently find robust social interaction deficits. Congenic *Fmr1* KO mice showed higher, indistinguishable, or lower levels of active direct social interaction than those of WT mice (28–34). These studies used 8- to 24-week-old mice. Our and others' data from 8-week-old mice indicate that *Fmr1* deletion has little or no effect on social approach and sociability at this age (32–34). Our observations suggest that detectable deficits in affiliative social interaction appear at 6 weeks of age. Moreover, we detected a statistically significant genotype difference after excluding cases where either stimulus or test mice exhibited aggressive behaviors or hyperactivity (see *Results*). It might be difficult to detect a subtle difference in affiliative social interaction in *Fmr1* KO mice if such confounding factors are not eliminated or not detectable in a three-chamber apparatus where aggressive and affiliative social approach cannot be separated and are equally recorded as more time in the vicinity of a caged stimulus mouse. In general, the rather weak defects in social behavior on a group basis in *Fmr1* KO mice are congruent with clinical observations that individuals with *Fmr1* deletions show incomplete penetrance for the full criteria of ASD diagnosis (35).

A novel aspect of the present study is that we identified neonatal vocalization sequences that best predict postpubertal social interaction scores. The number of flat->chevron and Markov probability of chevron->short sequence were significantly correlated with the postpubertal social interaction scores in a pooled data of vehicle-treated WT and KO mice (Supplemental Figure S9A). These parameters were also identified as the most robust predictors when the best predictors were explored within each genotype (see Figure 6). In other words, the level of these call sequences can provide insights into the future developmental trajectory of social interaction. This observation is not inconsistent with the hypothesis that a common developmental mechanism exists between neonatal social communication and postpubertal social interaction and that the normal postpubertal social interaction requires normal neonatal social communication, including the number of flat->chevron sequence. While the biological significance of these call sequences is not clear, we previously demonstrated that altered call sequences in a mouse mutant for another gene implicated in neurodevelopmental disorders lost the capacity to elicit maternal approach (11). More work is needed to critically evaluate whether the call sequence alteration of *Fmr1* KO pups contributes to a causal chain from the genotype of pups, impaired maternal care, and impaired development of social and cognitive capacities (17, 18).

While the number of flat->chevron was identified by a Lasso model as a predictor for the genotype (Supplemental Figure S9B), this variable did not clearly discriminate WT and KO genotypes (see Supplemental Table S2 and Supplemental Figure S9B). This weak discriminating power of the Lasso-identified variable is likely due to the overlapping nature of social interaction scores between WT and KO mice and a very weak difference in social scores between the two genotypes (Figure 5A). In more general

terms, the neonatal variables might be more suitable in predicting the continuous nature of postpubertal social scores than the categorical classification of the genotype.

Although the Lasso models extracted predictive neonatal call variables for the social interaction scores of each genotype with bumetanide treatment, no models achieved statistically significant correlation coefficients with the social interaction scores in bumetanide-treated WT and KO mice (Figure 6). One reason for the lack of correlations is that bumetanide treatment eliminated the effects of genotype on postpubertal social interaction by nonspecifically reducing the social interaction levels in WT and KO mice (see Figure 5). Thus, the effects of bumetanide on neonatal and postpubertal social behaviors are dissociated; bumetanide restored neonatal social communication but had nonspecific negative effects on postpubertal social interaction in WT and KO mice. This dissociation could be interpreted as suggesting that distinct mechanisms exist for neonatal social communication and postpubertal social interaction. More work is needed to critically evaluate the mechanistic origins of neonatal social communication and postpubertal social interaction.

This result does not support the hypothesis that bumetanide, when given around the time of birth, has beneficial effects on postpubertal social impairments in patients with fragile X syndrome. However, possibilities remain that lower doses of bumetanide have more specific ameliorative effects on the later social interaction or that this drug has beneficial effects on other types of social behavior such as social incentive learning (36) or maternal social behavior (11, 37) or nonsocial behaviors, including sensory hypersensitivity (38) and their cellular correlates (38, 39). Alternatively, distinct mechanistic origins and differential dependence on peripartum GABA signaling might exist for neonatal social communication and postpubertal social interaction. More work is needed to explore the possible effects of bumetanide administered at postnatal or earlier embryonic periods.

Although bumetanide is largely ineffective in alleviating ASD symptoms in humans, this treatment is generally started after a diagnosis of ASD at 2–3 years of age. If the perinatal period is the critical period for the therapeutic effects of bumetanide on neonatal social communication, as suggested by our observations, its therapeutic effects would be expected to be most robust when it is applied perinatally and its outcome is evaluated much earlier. Our observations and computational approaches provide a template for future work to explore the causally distinct neuronal substrates that subserve neonatal and later ASD-linked behaviors.

Methods

Mice

We used male FVB.129P2-Pde6^{+} Tyr $^{c\text{-}ch}$*Fmr1*^{tm1Cgr}/J mice (*Fmr1*$^{-/y}$, #004624, Jackson Laboratory, Bar Harbor, ME, USA) and their WT littermates. We chose male mice, as the symptoms of Fragile X syndrome in humans are more severe in males than females and female patients tend to exhibit a greater degree of interindividual variability (40). These mice were generated by crossing male *Fmr1*$^{-/y}$ mice (12–24 weeks old) with female *Fmr1*$^{+/-}$ mice (8–20 weeks old) as breeders. Their genotypes were determined by PCR using the following primers: 5′-TGTGATA GAATATGCAGCATGTGA-3′, WT forward; 5′-CACGAGACTAGTGAGACGTG-3′, homozygous forward; 5′-CTTCTGGCACCTCCAGCTT-3′, reverse for both genotypes.

This *Fmr1* mutant strain originally contained 129P2/OlaHsd alleles derived from E14 ES cells, but was converted to a congenic line through 11 generations of backcrossing of mutant mice to the FVB strain. This backcrossing eliminated both copies of the *Pde6b* mutant allele, a gene responsible for retinal degeneration; mutant mice do not suffer from retinal degeneration or blindness. Because this is a congenic strain, the confounding effects of unequally enriched 129P2/OlaHsd alleles surrounding the *Fmr1* gene in mutants and those of FVB alleles in their WT littermates are minimized (10).

Treatment

Female breeders were examined for a plug every day; when it was present, this was defined as 0.5 days post coitum (dpc). Pregnant females were then separated from their male partners. In the last week of pregnancy, cage bedding was not changed to minimize stress. Mice were randomly assigned to either the vehicle or bumetanide treatment group. At 18.5 dpc, bumetanide (#14630, Cayman Chemical Company, Ann Arbor, MI, USA) or vehicle was given in the drinking water, based on the expected volume consumption and the dam's body weight. This timepoint was chosen based on a published study (8). Our pilot study showed that mice consume at least 5 ml in 24 h, and the solution concentration was adjusted, based on the dam's weight, to greater than 2 mg/kg bumetanide. This regimen achieved the target dose when partum was confirmed at and after 19.5 dpc. The consumed dose of bumetanide ranged from 2.928 to 4.12 mg/kg (Supplemental Table S1).

Behavior

Male congenic *Fmr1*$^{+/y}$ (WT) and *Fmr1*$^{-/y}$ (KO) mice were used for behavioral analyses.

Ultrasonic Vocalization

When male pups reached P7, the cage containing the mother and the litter was transferred to the test room 30 min before testing. This developmental timepoint was chosen, as a previous study showed that *Fmr1* KO pups differed from WT pups in vocalizations at P7, but not at P4 or P10 (13). Moreover, another study tested the effects of bumetanide on vocalizations at P8 (8). Each pup was then moved to a test chamber (18 cm long × 18 cm wide × 30 cm high). Ultrasonic vocalizations were recorded for 5 min using an UltraSoundGate (Avisoft, Germany) connected to a computer equipped with Avisoft-RECORDER software (Avisoft). The sampling rate was set to 250 kHz (format, 16 bit). The low cut-off frequency was set at 10 kHz to reduce background noise outside the relevant frequency band. The frequency window for analysis ranged from 15 to 150 kHz. Call detection was performed using an automatic threshold-based algorithm and a hold time mechanism (hold time: 10 ms).

The weights of dams were measured to calculate the total dose of bumetanide they consumed. However, we did not measure the body weights of pups. The pups' body weights are not altered in this specific mouse model of fragile X syndrome (Jax#004624) compared with those of WT littermates, and no correlation of body weights with changes in call types was reported (41, 42).

Affiliative Social Interaction

Male mice that were tested for neonatal vocalization were sequentially tested for social interaction at 6 and 8 weeks of age. The test subjects and age-matched male *Fmr1*$^{+/y}$ non-littermates, used as stimulus mice, were habituated to the test room for 30 min. The test and stimulus mice were simultaneously placed in a test apparatus (20 cm long × 28 cm wide × 15 cm high; 50 lux), and their behavior was recorded for 5 min. Under this experimental condition, mice generally exhibit low levels of aggressive behaviors (12, 43–47). We used this naturalistic test instead of the three-chamber sociability test apparatus because the former is recommended for ultimate validation and the latter has many technical and interpretive issues (22, 48). Raters were blinded to the genotype and treatment until testing and scoring were completed.

Computational Analysis

Call Type Classification. Sonograms were inspected, and genuine noises were eliminated from the analysis. VocalMat software (26) was used to determine call types. This software has the lowest false positive and false negative rates among all call type classifiers (26). One modification was applied. VocalMat detects only salient elements in sonograms of what was classified as "harmonic" in our previous studies (11, 12, 45) and classifies such elements (e.g., step-up and step-down) as call types. To avoid these false negative cases, we manually inspected all call types and reclassified such cases as harmonic.

UMAP. We used the UMAP method (49) to independently classify call types and evaluate the impact of *Fmr1*$^{-/y}$ and bumetanide treatment on call types. UMAP is a dimensionality reduction technique based on Riemannian geometry and algebraic topology that helps cluster data with similar features. We utilized the Python library "umap-learn" (version 0.5.5), and the quantitative parameters of VocalMat were used as inputs. The quantitative parameters included the length (duration) and bandwidth of each call, the minimum and maximum frequency values in kHz

(min_freq_main, max_freq_main, mean_freq_main, min_freq_total, max_freq_total, mean_freq_total), and sound intensity in dB (min_intens_total, max_intens_total, and mean_intens_total) of various components of each call, where "main" and "total" designate the most intense wave component and all wave components, respectively, of each call. Additionally, the following parameters were set for UMAP calculation: random_state, 0 and n_neighbors, 30. The remaining parameters were left at their default values. After call clusters were identified by UMAP, we labeled each data point based on the modified VocalMat call type classification.

To test the validity and robustness of this approach, we created bootstrapped clusters from randomly chosen data points within each call type 2000 times. Each random selection generated the median data point for each call type. These 2000 median values per call type were plotted and compared with the positions of the data point distribution of each call in UMAPs. The positions of these median values clustered at the center of each call type of UMAP, thereby validating the UMAP data.

Call Sequence Analysis. As we reported previously (11, 12), we quantitatively defined a call sequence as a series of calls with intercall intervals below the intersection between the theoretical and observed distribution curves. Two calls with an intercall interval longer than the cross-point of the two curves were considered to belong to the last and first call of two distinct call sequences. Two-call pairs within so-defined sequences were then used for Markov modeling, using our published procedure (11, 12). There were 0 counts of some call pairs in some animals. A count of 1 was added to all call pairs of each animal to avoid 0 probabilities.

Lasso Model. We applied the Lasso regression model, following our previous method (12), to extract predictive variables of the number and proportion of each call type and two-call pairs within sequences for individual social interaction scores.

Statistical Analysis

All computer programs and data are available upon request. Analysis of variance (ANOVA) was used to compare more than two groups, and Student's unpaired and paired *t*-tests were used for comparisons of two groups. The normality and homogeneity of variance of data were evaluated using the Shapiro–Wilk and Levene tests, respectively. When either assumption was violated, data were analyzed using the Mann–Whitney test for unpaired data and the Wilcoxon nonparametric test for paired data. However, when a sample size was too small for the normality test *($n < 10$)*, the homogeneity of variance alone was used to decide whether to analyze data with parametric or nonparametric tests. The minimum significance level was set at $p < 0.05$. When more than two tests were applied to a data set, the significance level was adjusted using Benjamini–Hochberg correction at a 5% false discovery rate (FDR). All statistical values are provided in Supplemental Table S2; the original *p* values that remained significant after this adjustment are shown in figure legends. Excluded cases are detailed in the *Results* section.

Study Approval

Animal handling and use followed protocols approved by the Animal Care and Use Committee of Hirosaki University Graduate School of Medicine and were in accordance with the Rules for Animal Experimentation of Hirosaki University.

Data Availability

All raw data and supporting analytical code are available upon request. All statistical data are provided in Supplemental Table S2. All reagents and the mouse model are publicly available.

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Author Contributions

YS conducted and designed all experiments and wrote the manuscript. TT analyzed all data, made all figures, and wrote the manuscript. SS collected data. TH analyzed all data. KN oversaw the entire work, designed all experiments, and supervised YS and SS. NH oversaw the entire analysis of all data, supervised TT and TH, analyzed all data, made figures, and wrote the entire manuscript.

The manuscript has been read and approved by all authors. All authors take full responsibility for all data, figures, and text and approve the content and submission of the study. No related work is under consideration elsewhere. All authors state that all unprocessed data are available, and all figures provide accurate presentations of the original data.

Corresponding authors: Professor Kazuhiko Nakamura for any aspect of the work except for data analyses and Professor Noboru Hiroi for data analyses. These corresponding authors take full responsibility for the submission process.

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Author Disclosures

The authors have confirmed that no conflict of interest exists. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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RESEARCH ARTICLE

A novel neurodevelopmental-neurodegenerative syndrome that cosegregates with a homozygous SPAG9/JIP4 stop-codon deletion

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This report outlines the clinical features of a complex neurological phenotype shared by three siblings from a consanguineous family, characterized by intellectual disabilities, speech developmental delay, gait disturbance, cerebellar syndrome signs, cataracts, and dysmorphic features (square and coarse facial features, thick lips, deep palate, small and spaced teeth, low-set ears, strabismus, eyelid ptosis, and blond hair). Seizures and brain atrophy were later evident. In the cosegregation analysis, five family members and 12 family controls were studied by whole-exome and Sanger sequencing. The structural and functional effects of the protein were explored to define the mutated variant's potential deleterious impairment. Neurological and neuropsychological follow-ups and brain magnetic resonance imaging (MRI) were performed. We identified a single frameshift homozygous nucleotide deletion in the SPAG9/JIP4 gene (NM_001130528.3): c.2742del (p.Tyr914Ter), causing a premature stop codon and truncating the protein and originating a possible loss of function. The variant cosegregated in affected individuals as an autosomal recessive trait. The in silico protein functional analyses indicate a potential loss of 66 phosphorylation and 29 posttranslational modification sites. Additionally, a mutated protein structure model shows a significant modification of the folding that very likely will compromise functional interactions. SPAG9/JIP4 is a dynein-dynactin motor adapter for retrograde axonal transport, regulating the constitutive movement of neurotrophic factor signaling and autophagy-lysosomal products. Under stress conditions, it can potentiate this transport by the p38 mitogen-activated protein kinases (p38MAPK) signaling cascade. Both functions could be associated with the disease mechanism, altering the axon's development and growth, neuronal specification, dendrite formation, synaptogenesis, neuronal pruning, recycling neurotransmitters and finally, neuronal homeostasis—promising common mechanisms to be used with investigational molecules for neurodevelopmental diseases and neurodegeneration.

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Keywords: Intellectual disability, neurodevelopment, neurodegeneration, dementia, syndrome, axonal transport, retrograde signaling, signaling endosomes, MAPKp38 signaling pathway, dynein-dynactin motor adapter

Introduction

The molecular transport of molecules at the intracellular level is essential to a cell's development and survival (1). This challenge for neurons is permanent due to the everlasting and distant polarization between the axons and the neuronal body. However, distance is not the only challenge; localized delivery of presynaptic components must be successfully overcome to maintain synaptic transmission (2). To carry out this process, neurons use "axonal transport" to ship multiple substances that move along the microtubules of the axon in a bidirectional way (3, 4).

The kinesin complex drives anterograde movement transport (from the soma to the axon tip) and ships transport substances such as RNA, proteins, and organelles to growth cones and synapses (5). The opposite, retrograde movement (from the axon to the neuronal body), is dynein dependent and important for neurotrophic factor signaling (6), autophagy-lysosomal-autophagy, degradation, and nerve regeneration. The machinery for this axonal transport includes motor and microtubule proteins and essential adapters (7). Furthermore, protein kinase signaling pathways and posttranslational microtubule modifications are required to ensure efficient transport into neurons (2).

Alterations in axonal transport can emerge through several mechanisms: (1) defects in the organization of the cytoskeleton, (2) alterations in the binding of motor proteins to microtubules, (3) abnormal kinase or dynein activities, (4) destabilization of motor cargo binding, and (5) alterations in mitochondrial dysfunction energy (8). Thus far, inadequate and nonprogressive retrograde movements can disrupt synapses, axonal growth, plasticity, and neuronal homeostasis. Multiple neurological diseases are associated with axonal transport disorders (7, 9).

The JIP4 protein, encoded by the *SPAG9* gene, is a dynein-dynactin motor adapter that favors axonal retrograde flow. JIP4 is ubiquitously expressed, including the central and the peripheral nervous system (10). Protein expression studies in brain-derived neurodevelopment axons have shown high levels of JIP4, which are detected in lysosomal fractions and autophagic vacuoles (11). JIP4 promotes and stabilizes the association with dynactin while antagonizing kinesin binding (12). Thus, in a mutually exclusive manner, retrograde transport is activated. JIP4 is involved in postnatal brain development (13) and neuronal homeostasis by intracellular metabolites recycling to maintain neuronal homeostasis (14). In humans, SPAG9/JIP4 has been associated with the prognosis of different types of cancer (15) but never linked to intellectual disability and/or complex neurodevelopmental phenotypes.

This article describes three homozygous siblings with a mutant variant of the *SPAG9* gene, affected by a complex phenotype characterized by developmental and language delay, severe learning difficulties, and motor compromise impairment. The follow-up of this family for more than 10 years has also suggested a cognitive deterioration progressive impairment, suggesting a subsequent neurodegenerative process. Preliminary findings were presented on a poster (16).

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Methods

Study Site and Family

This study was carried out at the "Universidad de Antioquia," Medellin, Colombia, in collaboration between The Grupo de Neurociencias de Antioquia (GNA) and Genética Molecular (GENMOL) research groups. The bioethics committee of the university (Comité de Bioética, Sede Investigación Universitaria CBE-SIU) approved the protocol study. The subjects who were studied signed the informed consent form after a detailed explanation of the objectives and procedures of the study. In cases where the subject did not know how to sign, parents or representatives gave consent and signed the form. The family was identified in Antioquia, Colombia, in 2011 and clinically followed up with subsequent follow-ups until 2021.

Characterization of the Phenotype

The physician's team from the GNA carried out medical, neurological, and psychiatric follow-ups. Laboratory tests, brain magnetic resonance imaging (MRI), and neuropsychological evaluations were performed on the cases. Affected individuals were evaluated at a medical school pediatric-neurology meeting staff at the university hospital to discuss the complex phenotype and to consider some potential differential diagnoses. The neuropsychological evaluations were carried out using the GNA protocol, and the Wechsler Intelligence Scale (17) was additionally included. The severity of the cases was assessed using a comprehensive battery of structured and semi-structured clinical tools based on the DSM-5 criteria, according to IQ measures and functional performance scale assessment in daily life. Acquired dementia or cognitive impairment was diagnosed according to established standard criteria using neurological and neuropsychological evaluation tools and family reports. A more detailed description of these instruments is presented elsewhere (18).

Genetic Analysis

DNA extraction followed a standard extraction protocol with the peripheral blood samples using the salting out method (19), and the samples were stored at −20°C until sequencing. Macrogen performed the whole-exome sequencing (WES). The coding regions of the genome were sequenced by next-generation sequencing using the Illumina platform with an average coverage of 100X. The SureSelectXT Library Prep Kit (Target Enrichment System for Illumina Version B.2, April 2015) enriched the library (20). Sequencing was performed on a HiSeq 4000 instrument following the standard protocol to reach a 100X deep read average. The data were processed using the HCS software [HiSeq Control Software (HCS 3.3) version 3.3]. Sequencing data were converted to the FASTQ format using the Illumina package bcl2fastq module [version 2.16.0.10 from Illumina (21)]. The bioinformatic analysis was carried out with these data afterward.

Bioinformatic Analysis

The quality of the reads was evaluated with the fastqc v0.11.5 tool from the Babraham Institute, http://www.bioinformatics.babraham.ac.uk/projects/fastqc (22). Subsequently, the sequences were mapped to the hg19 reference genome available at the University of California, Santa Cruz (UCSC) website, http://hgdownload.cse.ucsc.edu/goldenpath/hg19/chromosomes/ using the Burrows-Wheeler Aligner, as implemented in the bwa-0.7.12 software, http://bio-bwa.sourceforge.net/ (23). Variant calling was performed using the Broad Institute's Genome Analysis Tool Kit GATK tool (GATK) v3.8-1 Best Practices for Germline SNP & Indel Discovery in the Whole Genome and Exome Sequence https://software.broadinstitute.org/gatk/ (24). For the process of marking duplicates, the Picard v1.119 tool https://broadinstitute.github.io/picard/ was used. Base recalibration processes (BQSR – base quality score recalibration), the search for single-nucleotide polymorphism (SNP)-type variants and INDELS (variant calling) and variant filtering (hard filtering) were carried out considering the protocol suggested by the tool GATK (Genome Analysis Tool Kit) from the Broad Institute (Best Practices for Germline SNP & Indel Discovery in Whole Genome and Exome Sequence) https://software.broadinstitute.org/gatk/best-practices/bp3step.php?case=GermShortWGS. Once the variants were identified, the annotation was carried out with the wANNOVAR (25) and Ensembl Variant Effect Predictor programs (26), which make use of the information collected in different databases and bioinformatic tools for the search of the allelic frequencies of the variants in the different continental populations such as 1000 Genomes, ExAC, ESP6500 and gnomeAD.

The clinical interpretation of genetic variants was considered by the guidelines proposed by the American College of Medical Genetics and Genomics and the Molecular Pathology Association (ACMG) (27). The tools InterVar (28) http://wintervar.wglab.org/ and VarSome (29) https://varsome.com/ were used to classify each of the identified candidate variants.

The variants were prioritized considering the following criteria: (1) Quality of the sequences: Depth across samples (DP>30). (2) Mode of inheritance: autosomal recessive. (3) Allelic frequency (MAF <0.01) in a population database (1000 Genomes, ExAC, ESP6500, gnomAD). We filtered only variants with a frequency less than 0.01 with a higher probability of causing major effects and rare Mendelian conditions. (4) Exomic variants or variants at splicing sites were included. (5) Pathogenic variants, probably pathogenic or variants of uncertain significance—VUS—were also included. (6) Pathogenicity predictors: variants cataloged as deleterious or possibly deleterious by more than three pathogenicity predictors, including, that is, SIFT, and Polyphem2 Polyphen2, and presenting with values higher than 14 by the CADD predictor, and (7) The potential relationship with the phenotype by considering Human Phenotype Ontology terms were evaluated.

Sanger Sequencing

The variant identified was replicated using Sanger sequencing in four samples: two affected individuals with DNA available and both parents. FinchTV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA), http://www.geospiza.com/Products/finchtv.shtml was used to evaluate the quality of the chromatograms. We used Aliview version 1.18 http://www.ormbunkar.se/aliview/ (30) and novoSNP version 3.0.1 http://www.molgen.ua.ac.be/bioinfo/novosnp (31) to identify and analyze candidate variant.

Functional and Structural Analysis

The bioinformatics study began with the analysis of the primary sequence in FASTA format to characterize the system from the potential perspective of posttranslational protein modifications considering the calculation of N-glycosylation of amino acids with amide groups in their side chain, from the probability as estimated by measured with the NetGlyc 1.0 server software.

Similarly, the interaction of O-glycosylation sites by the OH functional group was measured with the NetOglyc 4.0 server software. Likewise, the phosphorylation patterns were measured with the NetPhosK 3.1 server software for the amino acids whose side chain is serine, threonine, or tyrosine is present in the sequence of the evaluation of 17 kinases: ataxia-telangiectasia (ATM), creatine kinase I (CKI), creatine kinase II (CKII), Ca2+/calmodulin-dependent protein kinase II (CaM-II), DNA-dependent protein kinase (DNA-PK), epidermal growth factor receptor (EGFR), epidermal growth factor receptor-3 (GSK3), insulin receptor (INSR), protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), protein kinase G (PKG), ribosomal S6 kinase (RSK), Src family kinases (SRC), cell division cycle gene in Schizosaccharomyces pombe, named cdk1 in mammals (cdc2), cyclin-dependent kinase 5 (cdk5), and p38 mitogen-activated protein kinases (p38MAPK) (32–34).

To identify the three-dimensional structure of SPAG9, portions of the protein sequence were searched for in the Protein Data Bank. The analyzed structure by X-ray diffraction with a resolution of 1.80 Å was constituted by 70 amino acids (ID: 2 W83). This structural fragment was used as a template for the complete model (35).

To model the protein structures of the SPAG9 and the SPAG9 Tyr914Ter variants, three-dimensional SPAG9/JIP4 wild-type, as well as other likely structures, were obtained from the Alphafold repository built by the DeepMind-Evoformer module (36). Five models with a significant spatial arrangement convergence as measured by the local distance difference test (37) were chosen. The machine learning modeling allowed the reconstruction of the truncated protein encoded by the SPAG9 Tyr914Ter variant. From the five models, we selected the one with the best molecular score (38). The global alignment between the wild-type and the

Table 1. Clinical characteristics of affected individuals

	Case 1	Case 2	Case 3
Age at exam, years	36	34	32
Gender	M	F	F
Height (cm)	165	168	172
Body Mass Index	27.5	29.1	24
Head circumference (cm)	57	58	58
Facies and general characteristics	Blond hair, thick lips, coarse facial features, square face, deep palate, low-set ears, spaced teeth. Single palmar crease, pes cavus.		
Skin	Hyperpigmented macules in the periorbital region and face	Hyperpigmentation in periorbital region	–
Reported dyslipidemia	+	+	–
Other pathologies	–	–	Asthma, rhinitis
Delayed psychomotor development/ Intellectual disability	+	+	+
Language disturbance	+	+	+
Cataracts	+	+	+
Strabismus	+	+	+
Eyelid ptosis	Bilateral	Unilateral (left)	–
Seizures	Seizures from 34 years of age	–	–
Gait disturbance	Scoliosis. Walk with both feet apart and with the balls of the feet with external deviation	Impaired tandem gait, slight outward deviation of the balls of the feet	Impaired tandem gait
Parkinsonism	–	–	–
Cerebellar syndrome (intention tremor, dysdiadochokinesia, ataxia, dysmetria)	Ataxia	Dysmetria	Dysmetria and dysdiadochokinesis
Dystonia	+	–	–
Dysarthria	–	–	–
Impaired coordination	+	+	+
Pathological reflexes	Sucking reflex	–	Sucking reflex
Plantar reflexes	–	–	–
Urinary incontinence	+	–	–
Supranuclear palsy	–	–	–
Nystagmus	+	+	–
Psychiatric symptoms/behavior	He pulls out his nails and teeth. Aggressiveness.	Apathy, abulia, compulsion to eat	Irritability and disinhibition
Superior member			
Spasticity	–	–	–
Weakness	–	–	–
Hyperreflexia	–	–	–
Sensory disability	–	–	–
Lower member			
Spasticity	–	–	–
Weakness	–	–	–
Hyperreflexia	–	–	–
Sensory disability	–	–	–

mutant protein models was achieved with the Needleman Wunsch algorithm applied to the BLOSUM62 matrix and quantifying the RMSD standard deviation between the models. The three-dimensional models were pictured with the Chimera U.C.S.F visualizer v 1.1.1 (39).

Results

Clinical Description of the Affected Siblings

Two parents and three affected children constitute the nuclear family. Table 1 details the summary of clinical findings for each individual.

Case 1. Male, firstborn, 40 weeks, normal pregnancy. Spontaneous vertex delivery without any complications. Average weight at birth. At 1 year of life, significant motor delay development of bilateral eye cataracts. Head support is at 4 years old, but walking is difficult. He could not chew and swallow food until he was 6 years old, for which he was fed only with blended food. The first two words, "mama" and "papa," are at 4 years old. At the age of 30, the development of complete oral sentences begins. At 34 years old, development of generalized tonic-clonic seizures. Entirely dependent on all daily living activities but with behavior changes in recent years. He pulled out his nails and his teeth aggressivity. Functional scales have deteriorated over time.

Case 2. Female, second pregnancy product, without significant prenatal or perinatal history. Spontaneous vertex delivery without any complications. Hold the head and trunk up at 8 months old. Walking and pronunciation of first words around 16 months old. Severe learning and mental disability (only can sign). Currently, she pronounces only three words. She is independent in daily life functions and helps her mother with simple household tasks.

Case 3. The third average pregnancy female product. Spontaneous vertex delivery without any complications. Sitting at 10 months old, walking age at 15 months old, and first words at 24 months old. Developed bilateral cataracts at 4 years old. Mild motor and learning delay development.

Table 2. Summary of neuropsychological assessment of affected individuals

Cognitive function	Case 1	Case 2	Case 3
Mini-Mental State Examination (MMSE)/30	6	13	19
Orientation	Far below average	Far below average	Below average
Verbal fluency/denomination	0	7	In the average age and schooling
Word list memory/10	0	1	5
Evocation memory/10	0	1	3
Visual memory	Cannot take the test	Cannot take the test	Far below average
Attention	Cannot take the test	Cannot take the test	Cannot take the test
Executive function	Cannot take the test	Cannot take the test	Cannot take the test
Visuoconstructive function	Cannot take the test	Cannot take the test	Cannot take the test
Apraxia	+	+	+
Full scale IQ	45	45	45
Verbal IQ	48	50	49
Performance IQ	47	47	47

She works as a laundress in a dairy—recent years with marked disinhibition.

Neuropsychological Assessment. The three siblings were classified with moderate intellectual disability according to the DSM-5 criteria, and all three obtained an IQ score of 45 (Table 2).

Brain Imaging. Brain MRI was performed for all affected individuals. The MRI results show heterogeneity between all of them (Figure 1). There was no cortical dysplasia or polymicrogyria in any of the cases. Tractography by diffusion tensor imaging are normal. Cochlea, vestibule, and brainstem are normal. In all three siblings, there is mild microangiopathy (Fazekas scale grade 1 for deep white matter). Ventriculomegaly is predominantly left, with slight prominence of the sulci of the upper cerebellar vermis. It draws attention to decreased iron deposits in the globus pallidus and a slight increase in the putamen, being more significant in case 3. Case 1 attracts attention for microcephaly, decreased fronto-occipital diameter according to biometric parameters by age and sex (Figure 1A) (40). The hypoplastic corpus callosum (CC) without dysplasia, with a decreased thickness of the genu (40). Bilateral hippocampal malrotation with both collateral sulci are vertical (Figure 1B). Slight iron deposit in the putamen. Case 2 with lower limit frontal-occipital diameter and decreased thickness of the genu of CC, left hippocampal malrotation. In case 3, we additionally see malrotation of the left hippocampus. The putamen nucleus is seen with more significant iron deposits, and unlike the other cases, there is agenesis of the septum pellucidum (Figure 1B). Also, there is a very thin CC, mainly the splenium. Loss of subcortical volume, with a more dilated fourth ventricle and increased iron deposits in the dentate nucleus.

Identification, Clinical Interpretation, and Validation of Candidate Variants

The complete family included 36 members. Three affected siblings and their two parents were available for WES. We extend the analyses to the remaining family members, available in 17 individual exomes. A homozygous deletion in the SPAG9 gene *(NM_001130528.3): c.2742del (p. Tyr914Ter)* was identified in the three affected siblings-heterozygous in their parents and a maternal uncle. This variant has not been found in other family members and disparate populations according to 1000 Genomes, ExAC and GnomAD databases. It has not been found in exomes (coverage: 86.8) and genomes (coverage: 31.7), according to VarSome (29). Figure 2 shows the complete genealogy and the results of the Sanger sequencing of the four individuals.

Structural and Functional Analysis of the SPAG9/JIP4 Model Protein and Its Relationship with the SPAG9Tyr914

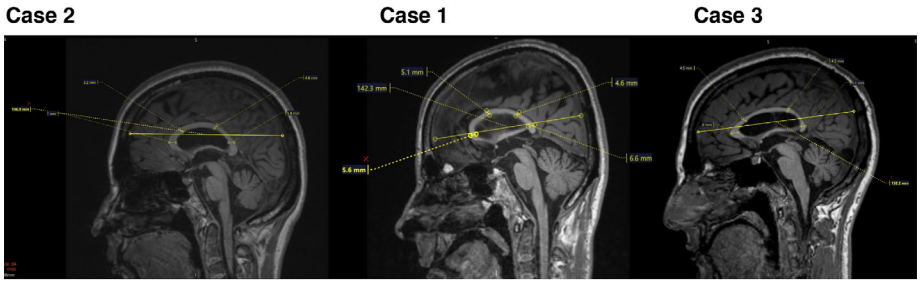
The SPAG9/JIP4 protein is expressed at the cytoplasmic level and in the cell lysosome, belongs to chromosome 17 and has a mass of 146,205 (Da). It has six isoforms, and the isoform with the highest frequency related to the interaction with kinesin is the isoform with a composition of 1321 amino acids (41–43). It binds to dynein and kinesin-1 in the leucine zipper II (JIP LZII) domain (Figure 3), allowing bidirectional vesicular transport along the microtubules and their dynamics (44).

The mutation found at position 914 is associated with a nucleotide change that produces a termination codon due to guanine (G) deletion. This stop codon prevents the protein from extending further and causing loss of structural information, but it directly affects the three-dimensional arrangement. Therefore, the protein presented changes in length and amino acid composition due to the lack of assembly of 407 amino acids. In the characterization of the system, the effect on the post-translational modifications that the protein can undergo and the changes it can generate were analyzed. Calculation of phosphorylation sites, O-glycosylation and N-glycosylation sites were made from the primary sequences to characterize the two proteins, as shown in Table 3.

For SPAG9wt, there are 257 phosphorylation sites in total (Table 4). The repeated numbers are due to their being phosphorylated by various kinases at the same position when they exceed the normalized phosphorylation value of 0.500. The mutation identified at position 914, which, in this case, produces a stop codon, means that the protein cannot extend further and loses structural information, which directly affects the three-dimensional arrangement. Regarding phosphorylation, due to the spatial effect from position 909, the phosphorylations considered up to the total SPAG9 protein with 1321 amino acids were lost (Table 4). These kinases contemplate positions with a triple possibility of phosphorylation, such as position 1188 with a nonspecific enzyme and two essential enzymes (protein kinase A and protein kinase C) involved in cell signaling. In the same way, position 1238 with the kinases p38MAPK, cdk5 and GSK3; position 1249 with a nonspecific enzyme, protein kinase C and cdc2; similarly, position 1262 with a nonspecific enzyme, DNA-PK and ATM. Finally, position 1264 has phosphorylation loss of a nonspecific enzyme, cdk5 and p38MAPK.

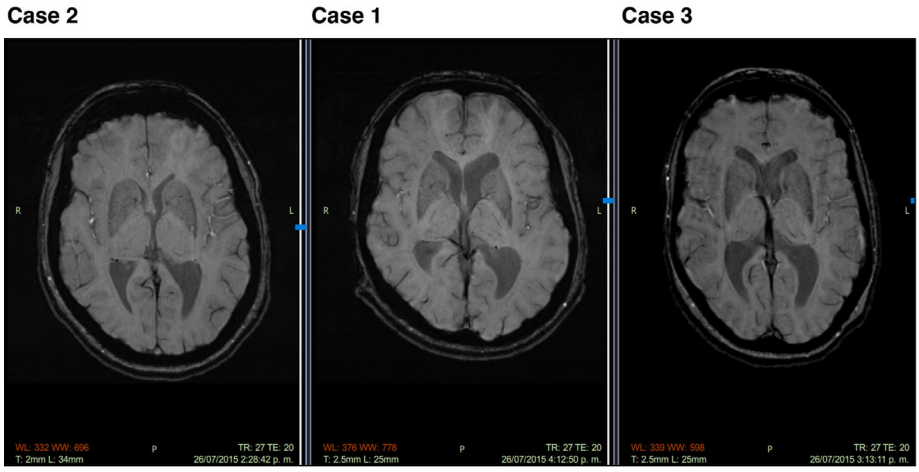
Regarding the O-glycosylations, 106 glycosylation sites were considered for SPAG9wt, which considered a probability greater than the threshold at 0.5. When the SPAG9 Tyr914Ter mutation occurred, there was a decrease in the total number of O-glycosylation sites, among which changes appeared in the N-terminal amino acid residue due to the effect of the change in spatial arrangement with position 16. These changes conceive effects by early protein termination and loss of glycosylation for positions 905, 909, 911, 915, 937, 938, 949, 1179, 1188, 1238, 1241, 1242, 1243, 1244, 1246, 1249, 1256, and 1262. These alterations in O-glycosylation affect protein polarity and the adhesion of other circulating protein systems. N-glycosylation was also a highly affected post-translational modification. Six modifications were made at positions 309, 565, 694, 830, 939, and 1176, but due to the mutation SPAG9 *Tyr914Ter,* only one modification was performed at position 309. Molecular modeling allowed us to obtain the SPAG9wt protein, as shown in Figure 4A and the variant with the stop codon in three-dimensional alignment with the native structure, in Figure 4B.

(A) 1.



	FOD	3rd Perc	GT	3rd Perc	BT	3rd Perc	IT	3rd Perc	ST	3rd Perc
Case 1	**142.3**	154	**5.6**		5.1		4.6		6.6	
Case 2	**146.9**	148.3	**7.0**	7.6	5.2	3.7	4.6	2.5	**5.8**	6.3
Case 3	152.2		8.0		4.5		4.2		**5.1**	

(A) 2.



(A) 3.

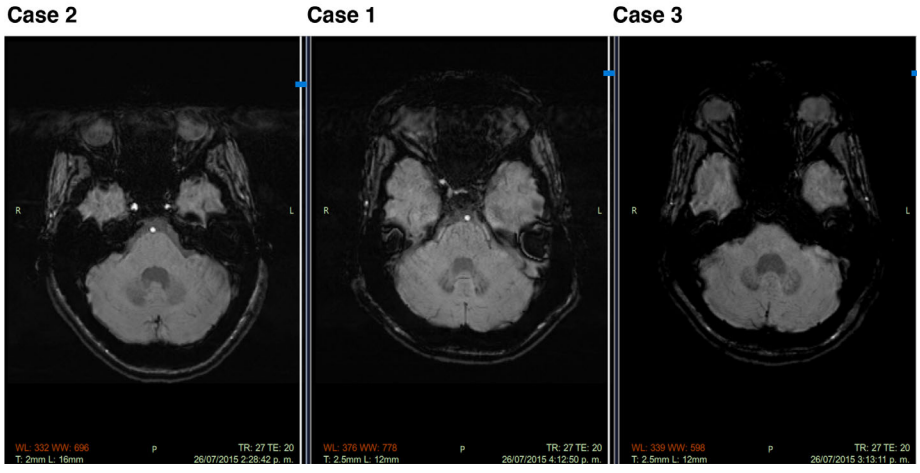
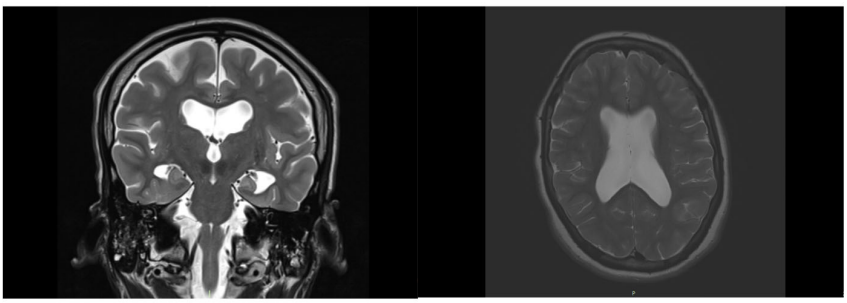


Figure 1. Findings reported on brain MRI of three siblings. (**A**) Comparison between the three cases according to 1. Measurements of the fronto-occipital diameter (FOD) and parameters of the CC (GT: Thickness of the genu, BT: Thickness of the body, IT: Thickness of the isthmus, ST: Thickness of the splenium) 2. Gradient of iron deposits in the putamen nucleus, from lowest to highest, with case 3 being highest. 3. Cerebellum and fourth ventricle. (**B**) 1. Bilateral malrotation hippocampal (Case 1). 2. Agenesis of the septum pellucidum (Case 3).

Discussion

This study reports a new syndrome with congenital alterations, neurodevelopment disorder, and neurodegeneration. The exomes analyzed from 17 family members, Sanger segregation analysis, and structural and functional evidence help determine possible pathogenic variants with significant effects. With the observations presented, we can conclude that this rare disease can be associated with the homozygous deletion of SPAG9/JIP4 gene. The family was identified from the rural area of northern Antioquia, Colombia, where a genetic isolate was previously reported, with a founder effect and several genetic disorders associated with a possible genetic bottleneck (45–47).

B 1. 2.

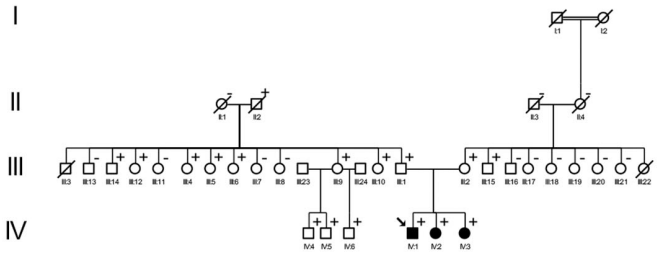


	Microcephaly	Hippocampal malrotation	Corpus callosum Hypoplastic	iron deposit (Putamen - dentate nucleus)	Cerebellar atrophy (Upper vermis)	Agenesis of the septum pellucidum
Case 1	+	Bilateral	+	+	–	–
Case 2	+	Unilateral	+	+	–	–
Case 3	-	Unilateral	++	++	+	+

Figure 1. (*Continued*)

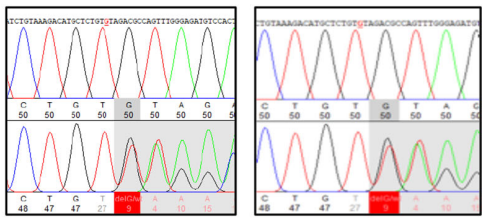
These three siblings present similar clinical characteristics. They had close scores on the intelligence scale (IQ) despite such a dissimilar Mini-Mental State Examination (MMSE), probably because of the differences in language. In other aspects, we can see heterogeneity in severity despite having the same mutation. The male case is the one who has the most severe motor disorder, with more incredible difficulty in language and functionality, in addition to severe psychiatric symptoms and facial dysmorphism. The younger sister (case 3) appears to have other essential alterations on MRI. However, functionally, she performed better in daily living activities and revealed minor differences in facial features.

A



B

III:1 and **III:2**. Normal phenotype heterozygous (G/–)



IV: 1 and IV: 2 Affected homozygous (–/–)

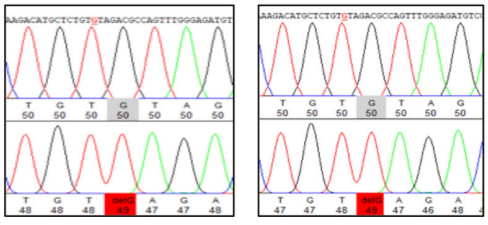


Figure 2. Family's pedigree and Sanger sequencing. (**A**) Pedigree shows four generations of the complete family. Squares are men, and circles are women. The arrow indicates the index case. Roman numerals are generations, and Arabic numerals represent the position of each individual in the family. Filled squares and circles represent affected family members. Slash (/) indicates a deceased person. The same line between two individuals represents consanguinity. Individuals with DNA samples are indicated with a plus (+) symbol. (**B**) Chromatograms of three subjects (parents and two siblings) were available and visualized with novoSNP (31). The reference sequence is visualized in the first trace. Variation is highlighted in red color. The unaffected parents III: 1 and III: 2 are heterozygous (G/–), and the two affected children IV: 1 and IV: 2 are homozygous (–/–) for the variant *(NM_001130528.3): c.2742del*.

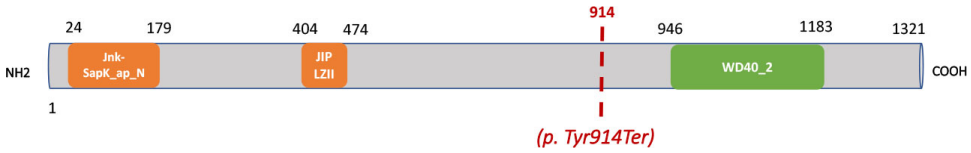


Figure 3. Localization of *(NM_001130528.3): c.2742del (p. Tyr914Ter)* in the domains of the SPAG9/JIP4 protein. Scheme of the SPGA9/JIP4 protein with the main domains and the location of the identified variant.

According to the MRI, microcephaly and bilateral hippocampal malrotation in case 1 were evident. Hippocampal rotation can begin between gestation weeks 21–32. Asymmetrical development is expected, with the right side faster than the left (48). A malrotation or incomplete inversion of the hippocampus can be expected in 19%. However, it can also be associated with structural variants associated with brain development that predispose to epilepsy to some extent (49). It is striking that this case has been the only one of the three siblings who has presented convulsive episodes after the age of 34.

The CC is the main structure that connects the cerebral hemispheres and integrates motor, cognitive, and sensory information. Morphological anomalies correlate with alterations in cognitive and behavioral development. Each subregion (genus, body, isthmus, and splenium) is associated with development and function in the cortex. We observed a decrease in the size of the genu and splenium. Genu are projections from the prefrontal cortex. The splenium has fibers from the occipital-parietal and temporal cortex (50). Full maturity of the CC appears to occur in early adulthood. It has been suggested that the increase in CC size at that age is related to the increase in axonal size (51). In all three cases, there is ventriculomegaly, possibly associated with subcortical atrophy and iron accumulation in the putamen and the dentate nucleus. Findings that could be associated with neurodegenerative changes. Iron is the most abundant metal in neurons; transport and storage failures are processes associated with neurodegenerative disorders (52). It is interesting to explore these findings further since they would provide clues to mechanisms not yet understood in dementia syndromes.

These cases were identified in the fourth decade of life, so we can observe the evolution of a neurodevelopmental disorder whose cause remains and appears to be progressing. In subsequent evaluations, all siblings had worsened in the functional scales (data not shown) and brain atrophy. Also noteworthy is the onset of seizures at age 34 and worsening motor and behavior impairment in case 1. Aspects that suggest possible degeneration after the developmental disorder.

Our findings conclude that deletion in the SPAG9 gene *(NM_001130528.3): c.2742del (p. Tyr914Ter)* generates changes in the protein regarding length and amino acid composition due to lack of assembly of 407 amino acids. This affects posttranslational modifications, affecting key sites for N-glycosylation, O-glycosylation, and phosphorylation. These three-dimensional effects and modifications are responsible for changing the chemical environment of the protein, which implies an alteration in cell function, either by activating a metabolic pathway or cell signaling, such as the interaction with cofactors and ligands such as dynein and kinesin-1, among others. The identified homozygous deletion produces a double truncated protein, with the absence of important kinase phosphorylation sites, including two positions for p38MAPK, in addition to the absence of 24 sites for O-glycosylation and five sites for N-glycosylation for its proper function.

JIP4 protein has two known molecular functions: (1) Dynein-dynactin motor adapter for retrograde flow. (2) Scaffold protein that potentiates the p38MAPK signaling cascade. Murine studies with a double knockout (KO) to JIP4 have shown neurodegeneration (11), but no previous cases with this phenotype associated with JIP4 mutations had been reported. The alterations in neurodevelopment and neurodegeneration seen in our patients could be explained by permanent alteration retrograde axonal transport and signaling deficiencies p38MAPK cascade.

p38MAPK regulates several cellular functions in the central nervous system, such as metabolism, secretion, migration, differentiation, apoptosis, and senescence (53). The cascade consists of three phosphorylation levels to activate p38, starting with a MAP kinase (MAP3K), then a MAP2K, and finally p38 MAP kinase. Protein kinases regulate axonal transport by phosphorylating motor and adapter proteins and cargoes directly and indirectly by modifying the microtubule network (54). MAPKp38 can negatively regulate axonal transport. In patients and mice with amyotrophic lateral sclerosis (ALS), overactivation of this signaling pathway produces alterations in axonal transport in the spinal cord (55). Scaffolding proteins, such as JIP4, recruit upstream MAP2K and MAP3K to enhance the activation of p38 kinases (10). JIP4 regulates retrograde and constitutive transport of lysosomes, but under stress conditions, it activates the p38 MAPK signaling pathway with posttranscriptional regulation of TMEM55B. TMEM55B recruits JIP4 to deliver dynein-dynactin to lysosomal membranes (56). Depletion of TMEM55B or JIP4 results in the dispersal of lysosomes toward the cell periphery (57). More research is required to understand alterations in JIP4 and their impact on regulating mTORC signaling (58).

The shortening of the protein was before the position of the WD40 domain (12). Proteins with this type of domain are associated with the function of interacting with other proteins (59). This domain would give its scaffolding function, which is fundamental to critical functions in signaling pathways and the gathering of multiple partners to facilitate concerted interactions and molecular functions. Functional studies are required to determine the implications of these findings for regulation, cell types or specific tissues, and machinery involved in each process where retrograde transport is involved.

The JIP4 homologous protein, previously identified as JIP3, overlaps with JIP4 in regulating axonal lysosome transport in neurons (60). According to previous studies, it has been suggested that JIP3 and JIP4 are functionally redundant and whose main difference is the expression of JIP3 only in neuronal cells (14). It has been hypothesized that if JIP3 is not expressed, JIP4 can replace JIP3 in the kinesin activation complex (14). De novo heterozygous mutations in the MAPK8IP3 gene encoding the JIP3 also show a phenotype of intellectual disability and brain abnormalities (61, 62). The reported variants are located in the four main domains of the JIP3 protein, including three mutations within the WD40 domain (62). Here, we report these first three cases with possible pathogenic mutations in JIP4, which demonstrate the importance of JIP4 at the brain level despite the presence of JIP3. This could agree with the statement that JIP4 has different functions than JIP3, and its presence is essential and not replaceable in certain brain functions or signaling pathways (10).

Retrograde endosomal signaling in neurons includes the following steps: internalization of ligand–receptor complexes into axon terminals, sorting of complexes into active signaling vesicles, transport along axonal microtubules to cell bodies, signaling endosomal and the dismantling of the complex (63). The central motor for retrograde transport is a cytoplasmic dynein complex composed of multiple subunits. This complex binds to microtubules and hydrolyzes ATP. However, on its own, it cannot carry out transport without dissociating from microtubules, so it depends on adapter proteins for efficient processivity (9).

How Does Defective Retrograde Axonal Transport Contribute to Neurodevelopmental Disorders?

Retrograde intracellular communication is essential for brain development and maintenance (2). The neurotrophin family of growth factors are synthesized and secreted away from neuronal cell bodies, propagate retrogradely along the axon to the body of the neuron and are required for proper neuronal survival, axonal growth, gene expression, neuronal

Table 3. Phosphorylation and posttranslational modifications sites in wild-type SPAG9 and SPAG9 Tyr914Ter

Protein	Phosphorylation sites	O-glycosylation sites	N-glycosylation sites
wild-type SPAG9	257 phosphorylation sites: Positions 9, 21, 21, 25, 30, 30, 32, 87, 109, 109, 109, 119, 119, 119, 123, 123, 126, 126, 143, 143, 183, 183, 183, 185, 185, 194, 194, 203, 203, 217, 217, 226, 226, 238, 238, 242, 244, 245, 245, 249, 251, 268, 268, 268, 268, 268, 272, 272, 275, 276, 276, 276, 279, 283, 292, 305, 305, 329, 330, 332, 332, 332, 339, 339, 339, 347, 347, 347, 348, 358, 363, 363, 364, 365, 379, 381, 381, 381, 387, 388, 391, 391, 418, 493, 493, 497, 504, 504, 504, 538, 538, 550, 550, 550, 551, 551, 551, 557, 561, 561, 562, 563, 564, 564, 564, 566, 566, 567, 567, 578, 582, 582, 583, 586, 586, 588, 588, 593, 593, 594, 594, 594, 595, 595, 595, 597, 611, 614, 617, 620, 620, 629, 683, 684, 705, 705, 710, 713, 728, 730, 730, 730, 732, 732, 733, 733, 756, 763, 764, 790, 804, 804, 806, 806, 813, 815, 822, 826, 829, 831, 832, 835, 837, 837, 848, 858, 858, 858, 858, 858, 861, 865, 865, 865, 879, 879, 887, 892, 895, 901, 901, 905, 909, 909, 909, 932, 935, 937, 944, 944, 966, 966, 967, 986, 996, 996, 1002, 1021, 1036, 1049, 1049, 1054, 1054, 1069, 1081, 1081, 1090, 1090, 1105, 1110, 1111, 1111, 1131, 1138, 1144, 1149, 1149, 1169, 1173, 1175, 1188, 1188, 1188, 1198, 1205, 1205, 1238, 1238, 1238, 1241, 1242, 1244, 1249, 1249, 1249, 1256, 1262, 1262, 1262, 1264, 1264, 1264, 1273, 1273, 1278, 1290, 1290, 1302.	106 O-Glicosilación sites: Positions 16, 25, 128, 183, 185, 190, 191, 194, 203, 226, 229, 238, 244, 245, 249, 251, 268, 272, 275, 276, 279, 280, 283, 287, 290, 292, 305, 325, 329, 330, 332, 358, 363, 364, 365, 367, 387, 493, 497, 504, 538, 551, 557, 561, 562, 563, 564, 566, 567, 582, 583, 586, 588, 593, 594, 595, 597, 604, 617, 620, 658, 705, 710, 724, 728, 730, 815, 822, 826, 828, 829, 831, 832, 835, 843, 848, 853, 857, 858, 860, 861, 865, 879, 887, 892, 895, 901, 905, 909, 911, 915, 937, 938, 949, 1179, 1188, 1238, 1241, 1242, 1243, 1244, 1246, 1249, 1256, 1262).	6 N-Glicosilación sites: Positions 309 NKSE (0,6999) 565 NTTK (0,5121) 694 NLSG (0,5093) 830 NSSA (0,5031) 939 NDSD (0,5029) 1176 NKTS (0,5162)
SPAG9 Tyr914Ter	191 phosphorylation sites: Positions 9, 21, 21, 25, 30, 30, 32, 87, 109, 109, 109, 119, 119, 119, 123, 123, 126, 126, 143, 143, 183, 183, 183, 185, 185, 194, 194, 203, 203, 217, 217, 226, 226, 238, 238, 242, 244, 245, 245, 249, 251, 268, 268, 268, 268, 268, 272, 272, 275, 276, 276, 276, 279, 283, 292, 305, 305, 329, 330, 332, 332, 332, 339, 339, 339, 347, 347, 347, 348, 358, 363, 363, 364, 365, 379, 381, 381, 381, 387, 388, 391, 391, 418, 493, 493, 497, 504, 504, 504, 538, 538, 550, 550, 550, 551, 551, 551, 557, 561, 561, 562, 563, 564, 564, 564, 566, 566, 567, 567, 578, 582, 582, 583, 586, 586, 588, 588, 593, 593, 594, 594, 594, 595, 595, 595, 597, 611, 614, 617, 620, 620, 629, 683, 684, 705, 705, 710, 713, 728, 730, 730, 730, 732, 732, 733, 733, 756, 763, 764, 790, 804, 804, 806, 806, 813, 815, 822, 826, 829, 831, 832, 835, 837, 837, 848, 858, 858, 858, 858, 858, 861, 865, 865, 865, 879, 879, 887, 892, 895, 901, 901, 905, 909, 909.	82 O-Glicosilación sites: Positions 25, 128, 183, 185, 190, 191, 194, 203, 226, 229, 238, 244, 245, 249, 251, 268, 272, 275, 276, 279, 280, 283, 287, 290, 292, 305, 325, 329, 330, 332, 358, 363, 364, 365, 367, 387, 493, 497, 504, 538, 551, 557, 561, 562, 563, 564, 566, 567, 582, 583, 586, 588, 593, 594, 595, 597, 604, 617, 620, 658, 710, 724, 728, 730, 732, 815, 826, 828, 829, 835, 853, 857, 858, 860, 861, 865, 879, 887, 892, 895, 901.	1 N-Glicosilación site: Position 309 NKSE (0,6928)

Comparison between primary sequences of two proteins (wild-type and mutated) according to phosphorylation, O-glycosylation and N-glycosylation sites.

Table 4. Positions phosphorylated by kinases that interact with *SPAG9wt* and the effect of *SPAG9Tyr914Ter*

Positions phosphorylated by kinases in SPAG9 (wild-type) are not present in SPAG9Tyr914Ter					
Position	**Amino acid**	**Enzyme**	**Position**	**Amino acid**	**Enzyme**
909	S	ATM	1149	T	PKA
909	S	DNA-PK	1149	T	cdc2
909	S	CKI	1169	S	PKA
932	S	unsp	1173	T	PKC
935	Y	SRC	1175	T	PKC
937	S	unsp	1188	S	unsp
944	Y	unsp	1188	S	PKA
944	Y	INSR	1188	S	PKC
966	S	PKA	1198	S	unsp
966	S	cdc2	1205	T	PKC
967	S	cdc2	1205	T	PKG
986	S	PKC	1238	S	p38MAPK
996	S	unsp	1238	S	cdk5
996	S	PKC	1238	S	GSK3
1002	S	unsp	1241	S	unsp
1021	T	DNA-PK	1242	S	unsp
1036	S	cdc2	1244	S	unsp
1049	S	unsp	1249	T	PKC
1049	S	PKA	1249	T	unsp
1054	T	unsp	1249	T	cdc2
1054	T	PKC	1256	S	unsp
1069	Y	unsp	1262	S	unsp
1081	S	unsp	1262	S	DNAPK
1081	S	PKC	1262	S	ATM
1090	S	unsp	1264	T	unsp
1090	S	PKA	1264	T	cdk5
1105	S	PKC	1264	T	p38MAPK
1110	S	RSK	1273	S	unsp
1111	T	PKC	1273	S	CKII
1111	T	unsp	1278	Y	unsp
1131	Y	unsp	1290	S	CKII
1138	T	PKC	1290	S	cdc2
1144	S	cdc2	1302	S	cdc2

Sixty-six lost phosphorylation sites of the mutated protein due to the spatial effect from position 909 (unsp = unspecific enzyme, S = serine, T = threonine, Y = tyrosine).

subtype specification, axon extension and branching, dendrite formation, neurotransmitters, synaptogenesis and synaptic function and axon regeneration (64).

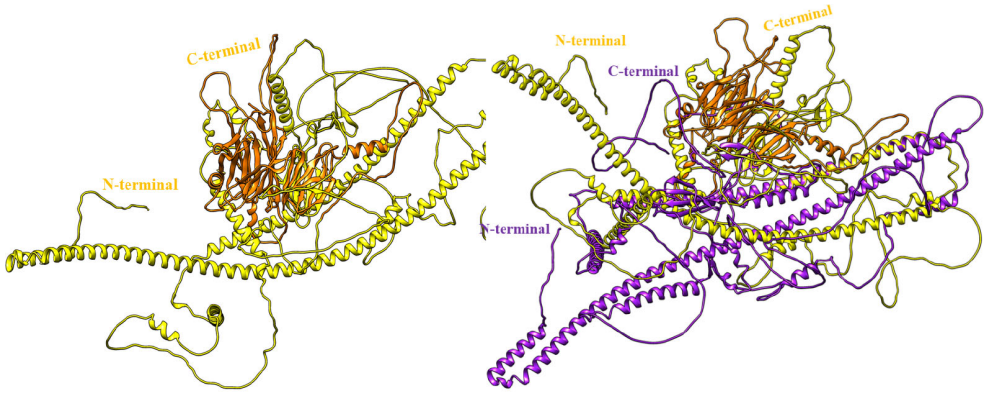
Synaptic dysfunction appears to be relevant in the absence of JIP4. Alterations in the JIP4 scaffold protein can directly impact synapses, mainly at the presynaptic but also at the postsynaptic levels (65, 66). The transport of neurotrophic factors necessary for the formation and maintenance of synapses at the presynaptic level and the postsynaptic level seems to involve the activity of lysosomes responsible for synaptic organization and neuronal pruning.

Recent report demonstrated that axonal lysosomal transport is altered by the loss of JIP4. JIP4/JIP3 could also be regulating the structure and dynamics of the neuronal cytoskeleton (58). Studies in mouse motor neurons concludes that adequate lysosomal activity is key to natural synapse elimination in mouse motor neuron (67). Authors have suggested possible common mechanisms for this regulation in nervous system both at the peripheral and central levels, during neuronal pruning and elimination of axonal connections to cause synaptic refinement (68).

Multiple Mendelian mutations, they have been associated with defects in motor proteins, adapters, or regulators of axonal transport (65). Some neurodevelopmental diseases involved specifically with proteins with functions in retrograde transport previously identified (9) are summarized below. Genetic alterations in the dynein cytoplasmic 1 heavy chain 1 (DYNC1H1 gene) with associated phenotypes: Charcot-Marie-Tooth disease, axonal, type 20, cortical dysplasia, complex, with other brain malformations 13 and spinal muscular atrophy, lower extremity-predominant 1. Mutations in regulators (NDE1 and BICD2) have been reported (69). NDE1 is associated to lissencephaly 4 (with microcephaly) and microhydranencephaly. BICD2-associated phenotypes are spinal muscular atrophy, lower extremity-predominant, 2A and 2B. PAFAH1B1 or LYS 1 is also an important gene required for dynein and microtubule dependent processes, and it is associated with lissencephaly type 1 (70). All of these phenotypes seem to involve more severe and earlier changes in brain development than the phenotype presented here, with clear malformations of cerebral cortical development (MCD) (71). In our cases, no MCD patterns were evident in the neuroimaging.

How Does Defective Retrograde Axonal Transport Contribute to Neurodegenerative Diseases?

Lysosomes recycle or eliminate damaged or misfolded proteins as they travel to the neuronal soma via retrograde axonal transport. Retrograde transport of lysosomes is recognized as an important regulator of autophagy. Autophagy maintains homeostasis and prevents the accumulation of toxic material within the cell. Neurons are particularly sensitive to this toxic accumulation (72). Multiple neurodegenerative diseases



A) SPAG9wt *B) SPAG9wt and SPAG9Tyr914Ter*

Figure 4. Representation of the three-dimensional model in tapes. (**A**) Region of the SPAG9wt protein after position 914 (in yellow and orange). (**B**) *SPAG9wt* and *SPAG9Tyr914Ter* (violet) proteins. There was no alignment of the structures. The effect of the charges of the amino acids was lost, affecting their folding and inducing changes in angles that alter the functional interaction.

have been related to defects in axonal transport, including Alzheimer disease (AD), Parkinson's disease, ALS, Huntington disease, frontotemporal dementia, Perry syndrome, Charcot-Marie-Tooth type 2B, among others (73). Autophagy is the process by which aged or toxic proteins and organelles are engulfed by the membrane forming an autophagosome that then fuses with a lysosome to form an autolysosome, with the aim of degrading the contents of the vesicle by lysosomal hydrolases. Lysosomal retrograde transport regulates autophagic flux by facilitating the formation of autophagosomes and fusion between autophagosomes and lysosomes (72). JIP4 phosphorylation acts as a switch that controls lysosomal distribution signaling pathways depending on the type of autophagy-inducing signal (72). Axonal transport of autophagosomes is regulated by JIP4 (74).

There is evidence of JIP3 the homolog of JIP4 and its role in neurodegeneration. The absence of JIP3 showed alteration in zebrafish retrograde transport and lysosome accumulation (75). And in dystrophic axons of the Jip3 KO mouse, immature lysosomes were found in the cell body (76). It has been seen that blocking retrograde transport leads to poor maturation and degradation of lysosomes contributes to their axonal accumulation and altered maturation in axonal inflammations of AD. In JIP3 KO mouse neurons, AD-like accumulations of lysosomes were identified (76) and in JIP3 +/− had worsening amyloid plaque pathology. These results show the importance of JIP3-dependent axonal lysosome transport in regulating amyloid precursor protein processing; however, in the 19 unrelated individuals with de novo variants in JIP3, with intellectual disability phenotype and brain malformations; no subsequent neurodegenerative changes or signs of developmental regression were described (61, 62). This characteristic was also not mentioned in a recent case report (77).

Our three cases are on average 34 years old and follow-up for more than 10 years with worsening cognitive and behavioral function has corroborated progressive cognitive deterioration. We did not find similar findings in the literature caused by dysfunction in the retrograde transport machinery or axonal transport in general, where cases with progressive phenotype, with neurodevelopmental disease and subsequent central neurodegeneration phenotype, are reported (9).

These observations generate multiple questions whose future answers could explain the described family's causal pathological mechanisms and many other diseases involved in cerebral retrograde transport, where development and degeneration tautologically converge.

Declaration of Possible Conflicts of Interests: All contributors have confirmed that no conflict of interest exits.

Author Contributions

N.A.-B. and C.A.V.-L. had full access to all the data in the study.

- Study concept and design: N.A.-B., J.T.-M., L.M., and C.A.V.-L.
- Acquisition, analysis, or interpretation of data: N.A.-B., J.T.-M., A.S.-O., A.M., M.P., and J.N.-T.
- Drafting the manuscript: N.A.-B. and M.A.-B.
- Critical revision of the manuscript for important intellectual content: M.A.C., W.R., G.P.C., and M.A.-B.
- Bioinformatic and structural analysis: N.A.-B., J.T.-M., and A.S.-O.
- Obtaining funding and Study supervision: C.A.V.-L. and M.A.-B.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Genomic Psychiatry

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RESEARCH REPORT

Treatment with shRNA to knockdown the 5-HT2A receptor improves memory in vivo and decreases excitability in primary cortical neurons

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Short hairpin RNAs (shRNA), targeting knockdown of specific genes, hold enormous promise for precision-based therapeutics to treat numerous neurodegenerative disorders. We designed an AAV9-shRNA targeting the downregulation of the 5-HT2A receptor, and recently demonstrated that intranasal delivery of this shRNA (referred to as COG-201), decreased anxiety and enhanced memory in mice and rats. In the current study, we provide additional in vivo data supporting a role of COG-201 in enhancing memory and functional in vitro data, whereby knockdown of the 5-HT2A receptor in primary mouse cortical neurons led to a significant decrease in mRNA expression ($p = 0.0007$), protein expression p-value = 0.0002, and in spontaneous electrical activity as measured by multielectrode array. In this regard, we observed a significant decrease in the number of spikes (p-value = 0.002), the mean firing rate (p-value = 0.002), the number of bursts (p-value = 0.015), and a decrease in the synchrony index (p-value = 0.005). The decrease in mRNA and protein expression, along with reduced spontaneous electrical activity in primary mouse cortical neurons, corroborate our in vivo findings and underscore the efficacy of COG-201 in decreasing *HTR2A* gene expression. This convergence of in vitro and in vivo evidence solidifies the potential of COG-201 as a targeted therapeutic strategy. The ability of COG-201 to decrease anxiety and enhance memory in animal models suggests that similar benefits might be achievable in humans. This could lead to the development of new treatments for conditions like generalized anxiety disorder, post-traumatic stress disorder (PTSD), and cognitive impairments associated with aging or neurodegenerative diseases.

Keywords: RNA interference, 5-HT2A receptor, memory enhancement, neuronal excitability, anxiety, cognitive impairment.

Introduction

Neurological disorders such as mild cognitive impairment (MCI) and chronic anxiety are a major public mental health challenge, affecting millions of people worldwide. MCI is often a transitional stage between healthy aging and dementia. Depending on the inclusion criteria, the prevalence of MCI has been estimated to be between 5.0% and 36.7% (1). According to a systematic review and meta-analysis, the overall pooled prevalence of anxiety in patients with MCI is approximately 21%. This prevalence rate varies based on the source of the sample and the method of diagnosis. For example, the prevalence of anxiety in community-based samples of patients with MCI is about 14.3%, while it is approximately 31.2% in clinic-based samples (2). Based on these statistics, we estimate that roughly 1.5–2 million Americans suffer from MCI with an underlying anxiety disorder. Currently, there is no single medication to treat both cognitive impairments and anxiety in this patient population.

Precision-based therapeutics such as RNA interference offer a promising new approach to treating neurological and neurodegenerative disorders. Short hairpin RNA (shRNA) represents one class of RNA interference molecules that has a mechanism based on the sequence-specific degradation of host mRNA through cytoplasmic delivery and degradation of double-stranded RNA through the RNA-induced silencing complex (RISC) pathway (3, 4). We designed plasmids containing the RNA instructions to construct a specific shRNA to silence the *HTR2A* gene (U.S. Patent Application No. 63/567,853). The *HTR2A* gene encodes for the 5-HT2A receptor, one of the 15 known serotonin receptor subtypes expressed in the brain, and is implicated in both anxiety disorders (5, 6) and memory (7–9). This plasmid contains a neuronal specific promoter, MeCP2 and is packaged within AAV9 viral particles. Intranasal treatment of this AVV9-shRNA (herein termed COG-201) in mice or rats significantly decreased anxiety and improved memory (10). In this study, we present further evidence supporting the memory-enhancing effects COG-201. We also provide functional data from experiments on primary cortical neurons taken from mice. Our results show that treatment with COG-201 leads to reduced spontaneous electrical activity in these neurons. This effect occurs specifically after reducing the expression of the 5-HT2A receptor. These findings bolster the potential of intranasal shRNA delivery as a non-invasive therapeutic method and establish a foundation for continued investigation into its role in treating anxiety and cognitive deficits linked to a spectrum of neurodegenerative diseases.

Methods

shRNA Design and AAV9 Vector Design

Construction of the mouse shRNA to target knockdown of the 5-HT2A receptor was as previously described (11). The mouse *HTR2A* gene consists of three exons that give rise to two major isoforms and is found on chromosome 13. The predicted binding region of the primary RNA transcript for this sequence is the beginning of exon 2, which would lead to the potential knockdown of all possible isoforms. The following sequence was used for assembly of the shRNA based on *in vitro* testing indicating a 77% knockdown:

GCTGAGCACATCCAGGTAAATCCAGGTTTTGGCCACGACTGACCTGGATTT
CTGGATGTGCT CAG

No knockdown was observed with the empty vector control or a scrambled shRNA control (Figure 2B). For validation and screening, knockdown was verified using HEK293 cells cotransfected with the cDNA plasmid containing the *HTR2A* gene target. For in vitro treatment of primary mouse cortical neurons, shRNA delivery subcloning of the shRNA was carried out in a modified pAAV *cis*-plasmid under the neuronal-specific promoter, MeCP2. The inclusion of the MeCP2 promoter is a crucial element design, as it ensures expression of the shRNA plasmid only in neuronal populations. A reporter gene enhanced green fluorescent protein was subcloned upstream of the shRNA sequence. AAV9 viral large-scale transfection of plasmids was carried out in HEK293 cells and purified through a series of CsCl centrifugations. Titer load (in genome copy number per mL, or GC/mL) was determined through quantitative real-time PCR, with typical yields giving $1–2 \times 10^{13}$ GC/mL. All AAV9 vectors were stored in phosphate buffered saline (PBS) with 5% glycerol at −80°C until used. Design, manufacturing, and purification of AAV9 vectors used in this study were performed by Vector Biolabs (Malvern, PA).

Novel Object Recognition Test

The object recognition task is used to assess short-term memory, intermediate-term memory, and long-term memory in rats and was performed as previously described (11). The task is based on the natural tendency of rats to preferentially explore a novel versus a familiar object, which requires memory of the familiar object. The time delay design allows for the screening of compounds with potential cognitive enhancing properties to overcome the natural forgetting process. Wistar male rats

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(12 animals per group) were randomly assigned to two groups consisting of vehicle (PBS) or COG-201 treated. Following administration of the vehicle or COG-201, rats were assessed in this task 3 weeks posttreatment and the discrimination index was calculated. To calculate the discrimination index, the following equation was used: (time exploring novel object-time exploring familiar object)/(time exploring novel object + time exploring familiar object), multiplied by 100 to convert to a percentage. The arena and objects were cleaned with 70% alcohol between each rat test session. These behavioral studies were performed by Neurofit SAS. All animal care and experimental procedures were performed in accordance with institutional guidelines and were conducted in compliance with French Animal Health Regulation. For all behavioral studies, animals were keyed, and data were blinded until the end of experiments.

Primary Mouse Cortical Neuron Cultures and Treatment with COG-201

Primary cortical neurons from fresh mouse brain embryos were isolated and plated onto coated 24-well plates at a density of 5×10^5 cells/well. Cortical neurons were maintained in Neurobasal-A Medium, supplemented with B27, Glutamax, and antibiotics (100 U/MI penicillin and 100 μg/mL streptomycin). Cultured neurons were incubated at 37°C and 5% CO_2 and half the media were exchanged with fresh, complete media every 3 days. On day 6 following plating (DIV 6), cortical neurons were treated exogenously with a stock concentration of COG-201 at 1×10^{13} (GC/mL) to a final multiplicity of infection (MOI) of 2×10^5. The MOI refers to the number of viral particles per neuron. Alternatively, cortical neurons were treated at the same MOI using AAV9-MeCP2-GFP-scrambled-shRNA representing the *HTR2A* target sequence but randomly scrambled. Cultured neuronal media was replaced with half, fresh, complete media every 3 days for 10 days (DIV 16) at which point cells were fixed for immunocytochemistry or analyzed for spontaneous electrical activity via MEA. Preparation and maintenance of primary mouse cortical neuron cultures was carried out by Creative Biolabs (Shirley, NY).

Ethical Animal Treatment Statement

Creative Biolabs complies with all provisions of the Animal Welfare Act and other regulations related to animals. Every individual involved in the care and use of laboratory animals fully understands the responsibilities, such as: avoids or minimizes discomfort, distress, and pain in experimental animals consistent with sound scientific practices; uses minimum number of animals necessary to obtain valid results. All experimental protocols were approved by the relevant Institutional Animal Care and Use Committee.

Immunohistochemical Fluorescence Microscopy

Immunofluorescence histochemistry was as previously described (10, 11). Briefly, following dehydration, 4 μm paraffin-embedded, sagittal sections were cut just lateral to the midline and used for immunofluorescence labeling. Briefly, all tissue sections were labeled with anti-GFP antibody (rabbit mAB #2956) 1:1,000 (Cell Signaling Technology, Inc., Danvers, MA, USA) or anti-5HT-2A receptor antibody (rabbit polyclonal, #24288) at 1:500 dilution (Immunostar, Hudson, WI). Secondary antibodies were conjugated to FITC or Cy3. DAPI was used as a nuclear stain. Whole slide scanning was performed using a Pannoramic Midi II scanner using a 40X objective lens with optical magnification of 98X, 0.1 μm/pixel. All sectioning, immunolabeling, and capturing of images was contracted out to iHisto (Salem, MA).

Immunocytochemistry Protocol

Cells were cultured under appropriate conditions before the immunocytochemistry procedure was initiated. For fixation, cells were treated with 4% paraformaldehyde prepared in 1x PBS. The fixation solution was preheated to 37°C prior to use. Cells were incubated with this solution for 10 minutes at room temperature to preserve cellular architecture and antigenicity. Following fixation, cells were washed thrice with 1x PBS, with each wash lasting for 3 minutes, to remove excess fixative. To permeabilize the cell membranes, 0.1% Triton X-100 (diluted in PBS) was added to the wells, and the cells were incubated for 15 minutes at room temperature. This step facilitates the entry of antibodies into the cells. Subsequently, cells were again washed three times with 1x PBS for 3 minutes each to remove the permeabilization agent.

The cells were then blocked with 500 μL of ready-to-use goat serum for 1 hour at room temperature to prevent non-specific binding of the primary antibodies. After blocking, the primary antibodies were diluted in the goat serum; GFP monoclonal antibody from mouse was diluted at 1:500, and 5HT2A antibody from rabbit at 1:100. The primary antibody solution was added to the wells, and the cells were incubated overnight at 4°C. The next day, the cells were washed three times with 1x PBS for 3 minutes each to remove unbound primary antibodies. The secondary antibodies were then prepared: AF488 Goat anti-Mouse IgG (H+L) and AF555 Goat anti-Rabbit IgG (H+L), both diluted at 1:400 in goat serum. The secondary antibody solution was added to the wells, and cells were incubated for 2 hours at room temperature in the dark to protect the fluorophores from photobleaching. After incubation with the secondary antibodies, cells were washed with 1x PBS to remove any unbound antibodies. Subsequently, nuclei were stained with a Hoechst solution, which was added directly to the wells. The cells were incubated in the dark for 5–10 minutes, followed by a final wash with PBS. Finally, the stained cells were imaged using appropriate fluorescence microscopy to detect the signals from the fluorophore-conjugated antibodies. Upon completion of imaging, the slides were sealed to prevent drying and to preserve the fluorescence for future analysis. Immunocytochemistry was performed by Creative Biolabs (Shirley, NY, USA).

Western Blot Analysis Protocol

Following treatment, proteins were extracted from cortical neurons the protein concentration was determined using a standard protein assay. Equal amounts of protein from each sample were then diluted with PBS to normalize the volume across all samples. The samples were mixed with loading buffer at a 1:4 volume ratio and denatured by heating at 100°C for 10 minutes. For electrophoresis, samples were loaded into a precast polyacrylamide gel alongside a molecular weight marker. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system. Posttransfer, the PVDF membrane was blocked in 5% non-fat milk prepared in Tris-buffered saline with Tween 20 (TBST) to prevent non-specific protein binding. Subsequently, the membrane was incubated with a primary antibody against the 5-HT2A receptor, diluted to a concentration of 0.3 μg/mL, and placed overnight at 4°C on a shaker. The membrane was washed three times with TBST for 10 minutes and then incubated with a goat anti-rabbit secondary antibody solution for 1 hour at room temperature on a shaker. For the detection of the antibody–protein complex, a chemiluminescent substrate was prepared and added to the membrane, which was then incubated for 5 minutes. The intensity of the bands was analyzed by densitometry to determine the relative amounts of the target protein present in the samples. Western blot analysis was performed by Creative Biolabs (Shirley, NY, USA).

Quantitative Real-time qPCR

Real-time qPCR was performed as previously described (11). Briefly, total RNA was extracted from primary cortical mouse neurons using a standard extraction protocol with TRIzol, dissolved in diethyl pyrocarbonate (DEPC)-treated deionized water and quantified. Following reverse transcription, qPCR was carried out using the following primers: Primer-F: 5′-AGAGGAGCCACACAGGTCTC-3′ and Primer-R: 5′-ACGACAGTTGTCAATAAAGCAG-3′. The relative expression was determined by calculating the $2^{-\Delta ct}$ value. The 2ˆ(−ddCt) value was then calculated and normalized to GAPDH for each treatment (AVV9-scrambled vs. COG-201). The RNA extraction and qPCR were performed by Creative Biogene (Shirley, NY, USA).

Multielectrode Array Analysis

MEA analysis was performed as previously described (11). Briefly, the microscope used was an Evos XL Core. Twenty-four-well MEA plates were coated with 500 μL 0.07% polyetherimide (PEI) and incubated for 1 hour. Plates were then washed four times in sterile deionized water and dried overnight in a biosafety cabinet. Primary cortical neurons from fresh mouse brain embryos were isolated and plated onto coated 24-well plates at a density of 5×10^5 cells/well. Cortical neurons were maintained in Neurobasal-A Medium, supplemented with B27, Glutamax, and antibiotics (100 U/MI penicillin and 100 μg/mL streptomycin). Cultured neurons were incubated at 37°C and 5% CO_2 and half the media were exchanged

with fresh, complete media every 3 days. Treatment (MOI of 2×10^5) occurred on day 6 for 24 hours at 37°C at which time the media was replaced with fresh, complete media. MEA analysis was then performed on day 16, 10 days after infection. MEA analysis was performed by Creative Biolabs (Shirley, NY).

Statistical Analysis

For PCR, and Western blot quantification, *t* tests for two independent means were calculated using excel software using a significance level set at 0.05 and one-tailed hypotheses. Microelectrode array data were analyzed via repeated measures ANOVAs using Statistica (Version 13.5, Tibco Software). All data used in these tests were checked and found to conform to parametric assumptions.

Acknowledgment of Generative Artificial Intelligence and Artificial Intelligence–assisted Technologies Used in Writing

In the course of this work's preparation, the author(s) employed ChatGPT 4 with the intent to improve readability and language. Upon utilizing this tool/service, the authors undertook comprehensive review and modification as necessary and assume full accountability for the content of the publication.

Results and Discussion

In a recent study, we have demonstrated a decrease in anxiety and improvement in memory following intranasal delivery of COG-201 in rats or mice (10). In that study, a novel recognition object test in normal, 2-month-old rats were carried out following treatment with COG-201. We reported a significant increase in both the contact-recognition index (92%) and time-recognition index (73%). In the current study, we now report a significant increase in the discrimination index in the novel recognition object test 3 weeks posttreatment with COG-201 (Figure 1A), and knockdown of the 5-HT2A receptor (Figure 1C) following intranasal delivery. The discrimination index is a measure used in the novel object recognition test to quantify the difference in exploration time between a novel and a familiar object. A positive discrimination index suggests that the animal spent more time exploring the novel object, which implies recognition of the familiar object and, thus, intact memory. Figure 1A indicates the vehicle-treated group exhibited a −28.9% discrimination index, which suggests that, on average, the rats spent more time with the familiar object than with the novel object during the retention test. One interpretation of these results is that the control group either did not remember the familiar object or there was an alternative factor at play (e.g., anxiety, stress). In contrast to the control group, the rats treated with COG-201 displayed a discrimination index increase of 22.5%. Thus, on average, treated rats dedicated more time to interacting with a new object rather than a familiar one, indicative of enhanced memory retention. The positive effects of COG-201 on memory retention are further highlighted when considering the significant negative discrimination index observed in the group treated with the vehicle alone. The contrast between the groups could suggest that COG-201 not only improves memory retention directly but may also improve it indirectly by reducing anxiety, or through a synergistic effect of both mechanisms.

These results, taken together with our previous findings support a memory enhancement action of COG-201. However, the missing component is functional data connecting the knockdown of the 5-HT2A receptor to the behavioral actions of COG-201. An additional aim of the current study was to provide functional data to support these behavioral findings. The serotonin 5-HT2A receptor is the major excitatory receptor subtype in the cortex. For example, this receptor has been linked with stress-induced dystonia, emphasizing its role in mediating neuronal excitability (12). In addition, the 5-HT2A receptor has been associated with excitatory effects in the neocortex and has been linked to working memory function by influencing both excitatory and inhibitory elements within local circuitry (13). Moreover, the 5-HT2A receptor has been found to directly stimulate key excitatory glomerular neurons in the olfactory bulb, further supporting its role in excitatory synaptic transmission (14). Overall, the 5-HT2A receptor plays a crucial role in memory, anxiety, and pain modulation, exerting excitatory effects in these processes. Therefore, we examined whether exposure of COG-201 to primary culture

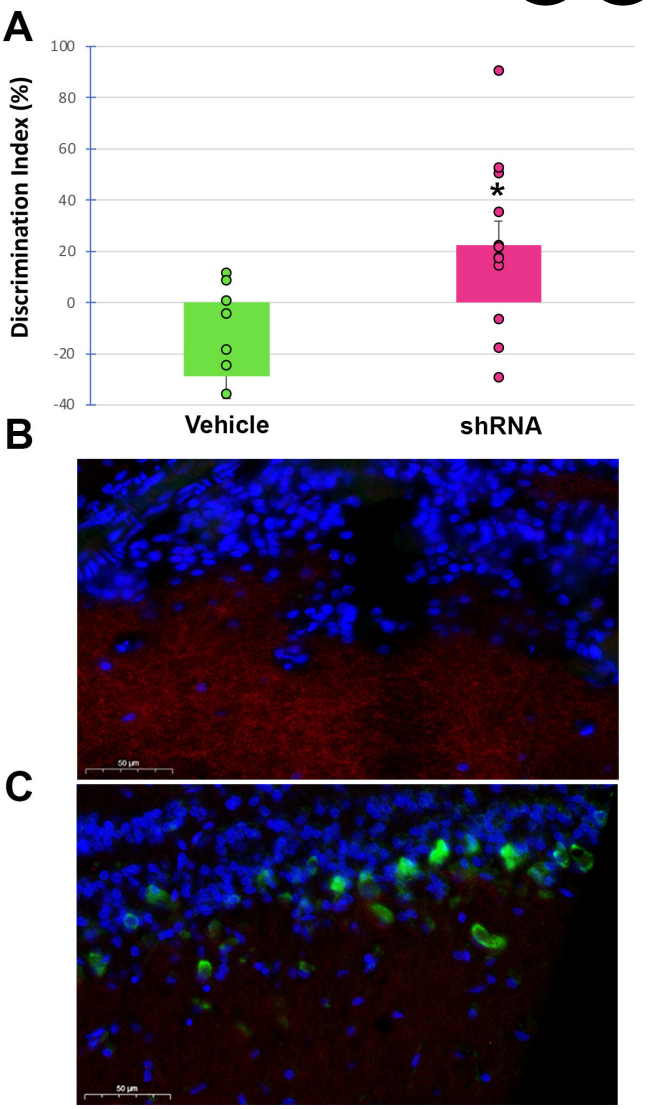
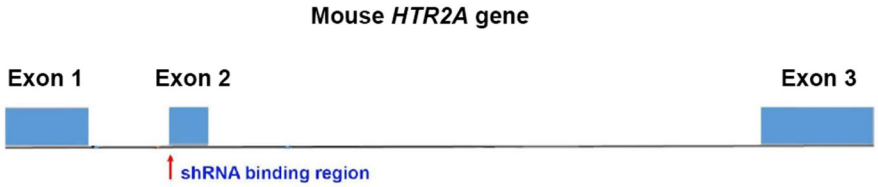


Figure 1. Intranasal adeno-associated virus delivery of AAV9-MeCP2-GFP-mouse HTR2A-shRNA improves memory in rats. The target sequence used to synthesize the shRNA is 100% conserved between mice and rats. To test whether COG-201 knockdown of the rat 5-HT2A receptor improves memory, Wistar rats (12 animals per group) were randomly assigned to two different groups consisting of vehicle-controls or COG-201. Following treatment on day 1, animals were assessed behaviorally 3 weeks later, and the discrimination index was calculated (see *Methods* for details). (**A**) At 3 weeks, there was a significant difference in the discrimination index between the two groups (p-value = .00025), with the vehicle controls at −28.8% (green bar) versus COG-201–treated +22.5% (pink bar). The green bar (labeled "Vehicle") shows the performance the control group. This group's discrimination index is around −20%, indicating a failure of preference for the novel object over the familiar one. This indicates poor performance in recognizing the new object. On the other hand, the pink bar (labeled "shRNA") represents the group of mice that received treatment with shRNA. Their discrimination index is around 30%, meaning they spent significantly more time exploring the novel object compared to the familiar one. This indicates better performance in recognizing the new object. (**B** and **C**) Representative, merged immunofluorescence image of vehicle-control animals depicting the presence of 5-HT2A receptor protein labeling (red fluorescence) within the olfactory bulb. The blue staining reflects nuclear staining with DAPI. As expected, there was no expression of GFP in vehicle controls (**B**) while strong GFP labeling was observed in cell bodies of neurons of shRNA-treated rats (**C**). Panel C also depicts a general lack of 5-HT2A fluorescence, supporting a knockdown of the receptor following COG-201 treatment. Images are representative of 3 separate rats for each group.

A



B

shRNAmir Vector	KD %	shRNAmir sequence
Empty Vector control	0%	
Scrambled shRNAmir control	0%	
shmir#1	68%	GCT GTCAATTGTCCAGTTCGAGGCTGTTTTGG CCAC TGACTGACAGCCTCGATGGACAATTGA CAG
shmir#2	61%	GCT GTGGAGATGAAGAATGGAGAGGGTTTTGG CCAC TGACTGACCCTCTCCACTTCATCTCCA CAG
Shmir#3	62%	GCT GAAACCCAGCAGCATATCAGCTGTTTTGG CCAC TGACTGACAGCTGATACTGCTGGGTTT CAG
Shmir#4 **	77%	GCT GAGCACATCCAGGTAAATCCAGGTTTTGG CCAC TGACTGACCTGGATTTCTGGATGTGCT CAG

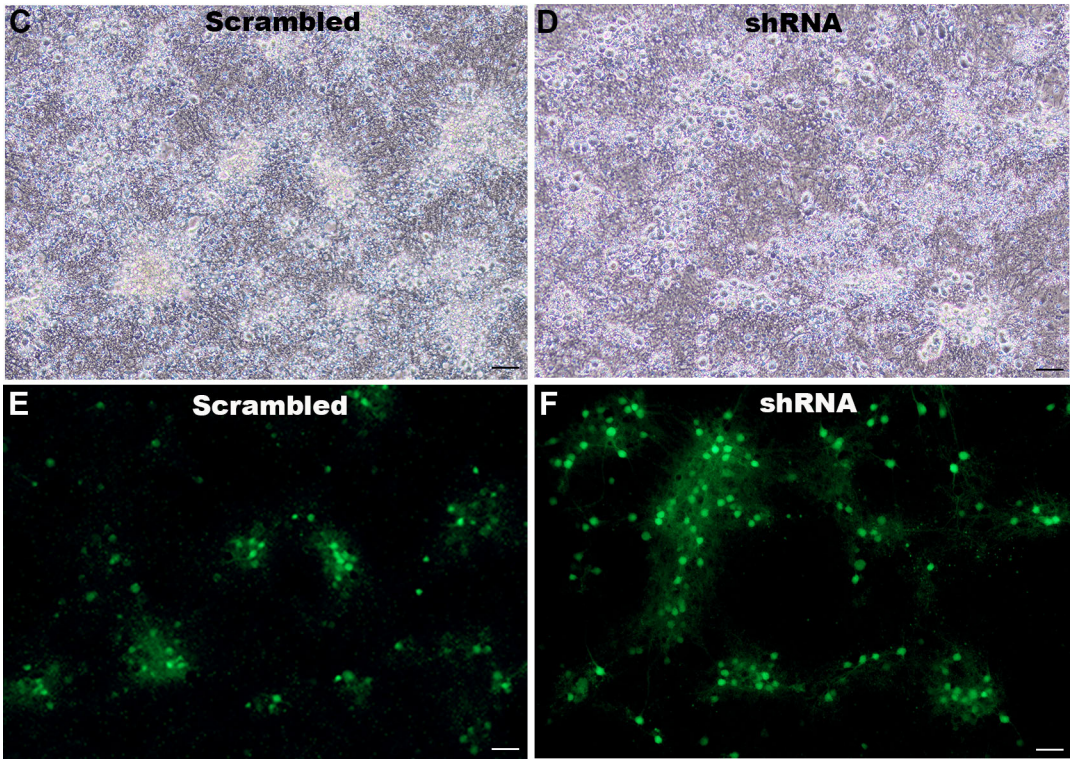


Figure 2. Targeting strategy to knock down the mouse HTR2A gene using shRNA and transduction efficiency in primary mouse cortical neurons. (**A**) Schematic displaying the *HTR2A* mouse gene encodes a single protein-coding transcript, *Htr2a-201*, located on chromosome 14 (11). The target sequence was constructed to recognize the beginning of exon 2 (red arrow, A). Knockdown of exon 2 prevent the production of all known full-length isoforms of the 5-HT2A receptors in mice. (**B**) To verify knockdown, in vitro experiments were undertaken using four different shRNAs, with shmir#4 giving the largest percent knockdown of HTR2A mRNA (77%) compared to 0% knockdown for either the empty vector control or a scrambled shRNA control. (**C**–**F**) Transduction efficiency of AAV9-mHTR2A-shRNA in mouse primary cortical neurons. (**C** and **D**) depict representative microscopic images in mouse neurons following a 10-day treatment with scrambled AAV9 shRNA-AAV9 viral particles (**C** and **D**) or mHTR2A shRNA-AAV9 viral particles at a MOI of 2×10^5 (**D** and **F**). Panels C and D represent bright-field images while Panels E and F are fluorescence images representing green fluorescence protein expression. For both constructs, strong GFP expression was observed.

neurons would lead to a decrease in electrical activity as measured by MEA. In this case, we measured the spontaneous activity of networks following treatment by recording field potentials. The advantage of MEA is that it can generate high-throughput readout of neuronal populations with the placement of multiple electrodes recording all at once rather than individually.

As an initial approach, we determined the relative transduction efficiency in primary cultured mouse neurons following *in vitro* treatment with either COG-201, or a scrambled AAV9-shRNA version. Figure 2A outlines the *HTR2A* gene targeting strategy, where shRNA is designed to bind at the start of exon 2, effectively halting the synthesis of all known full-length 5-HT2A receptor isoforms. Figure 2B demonstrates the efficacy of our targeted silencing approach, where shmir#4 induced a 77% decrease in HTR2A mRNA levels. This reduction is in stark contrast to the negligible impact observed with the scrambled shRNA control. The comparison was made in HEK293 cells that were cotransfected with a cDNA plasmid specifically engineered to contain the *HTR2A* gene sequence targeted by the shRNAs. As expected, following treatment of mouse primary cortical neurons, high transduction efficiency of AAV9-mediated shRNA delivery for both the AVV9-scrambled shRNA (Figure 2E) and COG-201

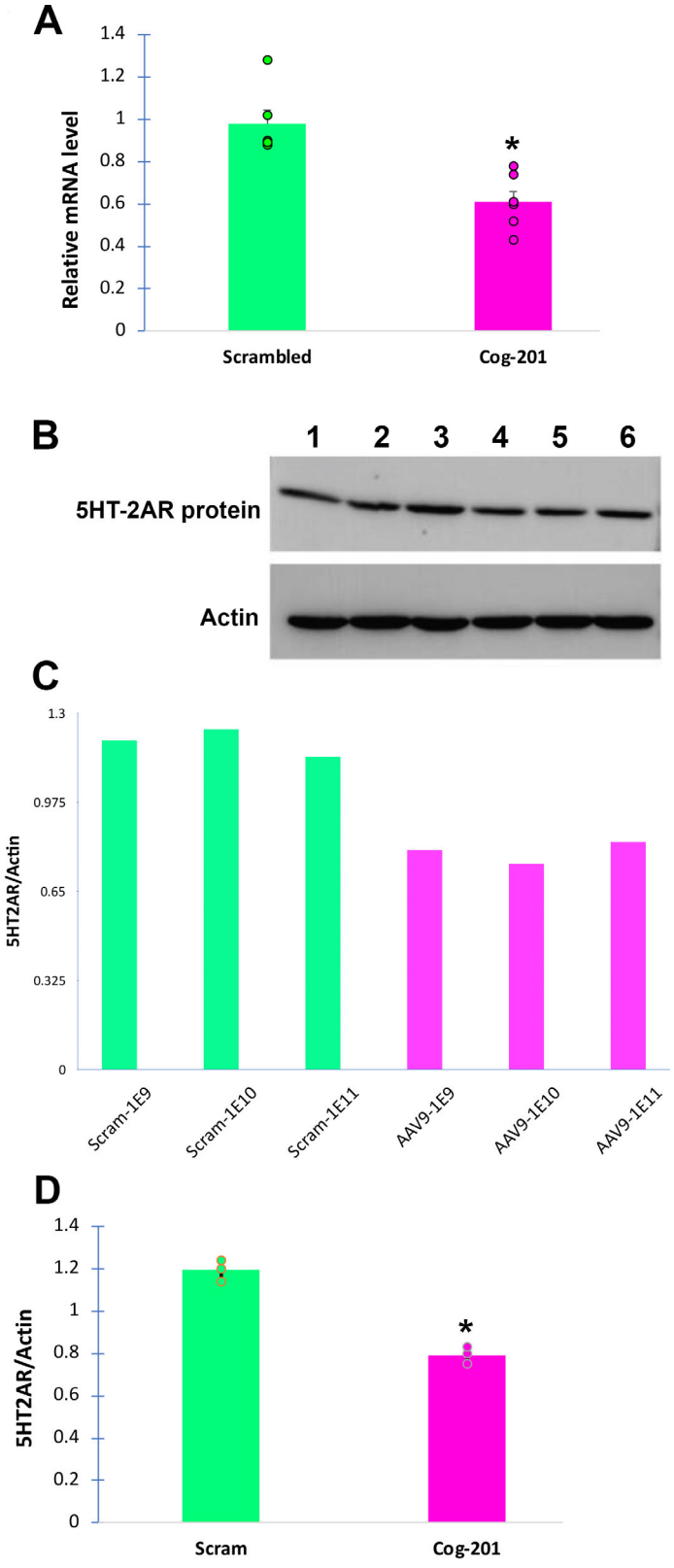


Figure 3. Treatment of mouse primary cortical neurons with shRNA leads to knockdown of the 5-HT2A receptor. Primary cortical neurons were treated on day 6 of plating with scrambled AAV9 shRNA-AAV9 viral particles (**A**) or COG-201 (**B**) at MOI of 3×10^5 for 10 days (day 16, 10 dpi) at which time, mRNA was isolated for real-time qPCR experiments. Results display the relative change in expression using GAPDH (*Continued on the next column*) (*Continued*) as an internal control. Real-time qPCR results represent a total of three separate treatments for each condition in which cells were pooled and frozen at −80°C. PCR experiments were performed in triplicate. The results indicated a significant 38% decrease in HTR2A mRNA expression as compared to vehicle controls, $p = .0007$. (**B**–**D**) Cortical neurons were treated at various concentrations and cell homogenates were prepared for Western blot analysis. Transferred membranes were incubated with 0.3 μg/mL of anti-5HT2A receptor antibody overnight at 4°C followed by goat anti-rabbit secondary antibody for 1 hour at room temperature. Panel B displays the results indicating 5-HT2AR protein band in scrambled-treated neurons (lanes 1–3) or in AAV9-shRNA–treated neurons (lanes 4–6). Densitometry analysis indicated a decrease in band intensity for COG-201 treated neurons (**C**). In panel D, data from lanes 1–3 and 4–6 were combined and the resulted data indicated an overall 34% decrease in 5-HT2A receptor protein in treated neurons versus scrambled controls (p-value = .0002).

(Figure 2F) was observed by fluorescence microscopy, as indicated by robust GFP expression.

We next determined the extent of 5-HT2A receptor knockdown by real-time qPCR or Western blot analysis (Figure 3). In this investigation, primary cortical neurons underwent treatment with shRNA to assess the knockdown of the 5-HT2A receptor expression, as illustrated in Figure 3. Neurons treated with COG-201 exhibited a significant 38% reduction in HTR2A mRNA expression as compared to the scrambled AAV9 shRNA-AAV9 controls, a finding confirmed by real-time qPCR with GAPDH as a reference ($p = 0.0007$). This knockdown of HTR2A mRNA was further substantiated at the protein level through Western blot analysis. After incubation with anti-5HT2A receptor antibody, the resulting combined densitometry results revealed a corresponding 34% decrease in 5-HT2A receptor protein levels in neurons treated with COG-201, as compared to scrambled controls (Figure 3C and D), (p-value = 0.0002), thus confirming the knockdown at both transcriptional and translational levels.

Further confirmation of 5-HT2A receptor knockdown by COG-201 was obtained by immunocytochemistry. Neurons treated with scrambled AAV9-shRNA viral particles showed strong expression of the 5-HT2A receptor protein, as evidenced by the robust red fluorescence (Figure 4A and D). In contrast, neurons treated with COG-201 exhibit a marked decrease in 5-HT2A receptor expression (Figure 4E and H), indicating successful receptor knockdown. Collectively, the results presented in Figures 3 and 4 confirm the successful targeting and subsequent knockdown of the 5-HT2A receptor by COG-201, establishing the rationale for the next phase of the study, where we aimed to elucidate the implications of 5-HT2A receptor knockdown on neuronal excitability employing MEA analysis.

To accomplish this, primary cortical neurons were treated on day 6 with COG-201 or the scrambled AAV9-shRNA version and spontaneous electrical activity (MEA measurements) were recorded 10 days later. Several parameters were measured including (a) the number of spikes (Figure 5A), which is defined as the total count of action potentials (spikes) recorded by the MEA over a 5-minute period, where each spike is a brief electrical impulse that represents a single neuronal firing event; (b) the mean firing rate (Figure 5B) defined as the average rate at which a neuron fires action potentials (spikes) measured in hertz (Hz); (c) the number of bursts (Figure 5C), defined as a cluster of action potentials (spikes) that occurs in quick succession, followed by a period of silence; (d) the synchrony index (Figure 5D), defined as how in sync the firing of different neurons or groups of neurons is with values closer to 1 indicating strong synchrony; (e) number of network bursts (Figure 5E) representing coordinated activity across the neural network, thought to be crucial for various neural processes, including learning and memory and are indicative of the network's ability to engage in coordinated processing and communication; and finally, (f) the number of active electrodes (Figure 5F). A higher number of active electrodes typically suggests a more widespread or synchronized activity across the network, indicating robust interneuronal communication and network integration. To summarize the results in Figure 5, we observed a significant decrease in the

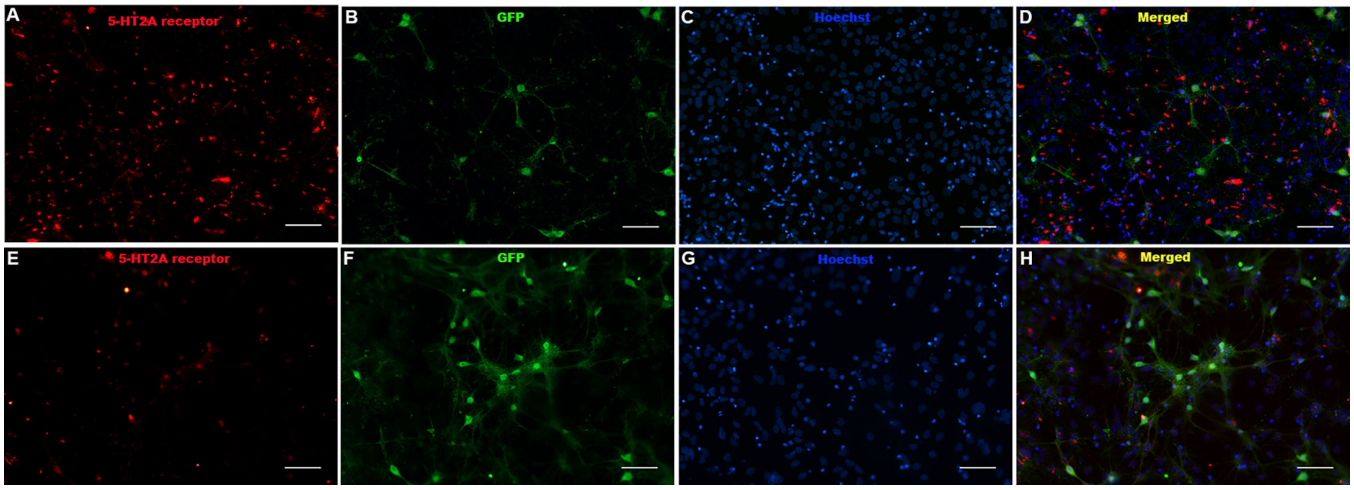


Figure 4. Treatment of mouse primary cortical neurons with COG-201 leads to knockdown of the 5-HT2A receptor. Representative immunofluorescence images in mouse neurons following a 10-day treatment with either scrambled AAV9 shRNA-AAV9 viral particles (**A**–**D**) or COG-201 at MOI of 3×10^5 (**E**–**H**). Green fluorescence represents green fluorescence protein expression detected using a GFP monoclonal antibody (mouse, 1:500) (**B** and **F**), while red fluorescence is indicative of 5-HT2A receptor protein following immunocytochemistry using an anti-rabbit 5-HT2A receptor antibody (Immunostar, 1:100). Panel A and D display robust expression of the 5-HT2A receptor protein in neuronal cells following treatment with the scrambled control. In contrast, a significant reduction in 5-HT2A fluorescence intensity was evident following treatment with COG-201 (**E** and **H**). Panels C and G represent Hoechst nuclear labeling while panels D and H represent merged images. All scale bars represent 50 μm.

number spikes, mean firing rate, number of bursts, and synchrony index but an increase in the number of network bursts following treatment with COG-201 in non-stimulated neurons. These data could be interpreted to suggest that a reduction in overall excitability supported the actions of COG-201 on knockdown of the excitatory 5-HT2A receptor. In addition, the significant decrease in the number of bursts of isolated neurons and in the synchrony index suggests that neurons with a reduced expression of 5-HT2A present with a lower frequency of spontaneous electrical activity (from 12 to ~6 Hz). On the other hand, a significant increase in the number of network bursts, that is, a coordinated electrical spiking within groups of neurons, is indicative of collective network behavior. An increase in network bursts amidst decreases in individual spikes, mean firing rate, and synchrony suggests that while overall activity and global baseline coordination are reduced, these effects may be compensated by increasing the instances of global synchronization across neurons forming a new network (15). The presence of desynchronized non-burst firing and partially synchronized bursts in developing networks of cortical neurons supports the notion of network compensation and adaptation (16), suggesting a Hebbian field. Synchronization of bursting neurons is a critical factor in understanding network behavior, and it has been shown that burst firing can promote synchronization between interconnected loci in central nervous system networks (17). In summary, the observed changes in multielectrode array (MEA) recordings following the treatment of primary cortical mouse neurons with COG-201 suggest a compensatory mechanism. Specifically, an increase in network bursts, despite decreases in individual spikes, mean firing rate, and synchrony, may indicate enhanced global synchronization within newly forming neuronal networks. This contrasts with the spontaneous global synchronization observed in all neurons treated with scrambled-AAV9-shRNA, which suggests the absence of distinct neuronal networks. This adaptive response at the network level may have implications for conditions such as anxiety and memory impairments.

An important caveat of the current study is connecting the MEA data with the underlying behavioral observations of a decrease in anxiety and improvement in memory. In the current study, we focused on cortical neurons; however, important neural networks implicated in memory and anxiety are found in the hippocampus and other subcortical areas including the interpeduncular nucleus (IPN). Previously, we identified a general pattern of guide RNA expression in the CA2/CA3 regions of the hippocampus in mice treated with CRISPR/Cas9 (10). Additionally, there was a noticeable reduction in 5-HT2A receptor expression, particularly in the apical dendrites of glutamatergic neurons. Previous research has documented the presence of 5-HT2A receptor mRNA in the CA3 region of the hippocampus (18, 19). As the 5HT-2A receptor is excitatory, its downregulation in the apical dendrites may enhance memory by influencing hippocampal neuronal oscillatory rhythms (20, 21). In the context of the current study, projections to the apical dendrites of CA3 pyramidal neurons could originate from the cortex, particularly the entorhinal cortex, which is essential for sensory integration and memory formation. In terms of how our molecular findings could connect behaviorally to enhanced memory, it is essential to consider the broader neural circuits involved. The hippocampus plays a critical role in memory formation and anxiety regulation. Previous studies, including our own, have shown that the CA2/CA3 regions of the hippocampus are vital for these processes. Our findings have demonstrated a reduction in 5-HT2A receptor expression in the apical dendrites of glutamatergic neurons. The 5-HT2A receptor is known to be excitatory, and its downregulation can modulate hippocampal neuronal oscillatory rhythms, which are crucial for memory consolidation. The CA3 region, in particular, receives projections from the entorhinal cortex, which is essential for sensory integration and memory formation. The findings of reduced 5-HT2A receptor expression suggest a potential mechanism where altered serotonergic signaling in the hippocampus can influence cortical inputs, thereby enhancing memory functions. This aligns with the observed behavioral improvements in the current study.

We have also previously demonstrated a decrease in 5-HT2A receptor density in the IPN (10), an area implicated as a major connectome for stress-mediated pathways (22). Serotonergic cortical neurons are known to connect to the IPN via the habenula pathway (23, 24). Therefore, the downregulation of the 5-HT2A receptor in IPN neurons, along with a corresponding decrease in electrical excitability, could lead to a reduction in anxiety-related behaviors. Together with the effects of these cortical projections, however, we cannot exclude a possible modulatory role of 5-HT2A receptors expressed on local intrahippocampal interneurons that regulate the firing of pyramidal hippocampal subfield neurons, a role not specifically addressed in the current study. Previous studies have demonstrated that 5-HT2A receptors on GABAergic interneurons stimulate GABA release, and thereby have an important role in regulating network activity and neural oscillations in the amygdala and hippocampal region (25–27).

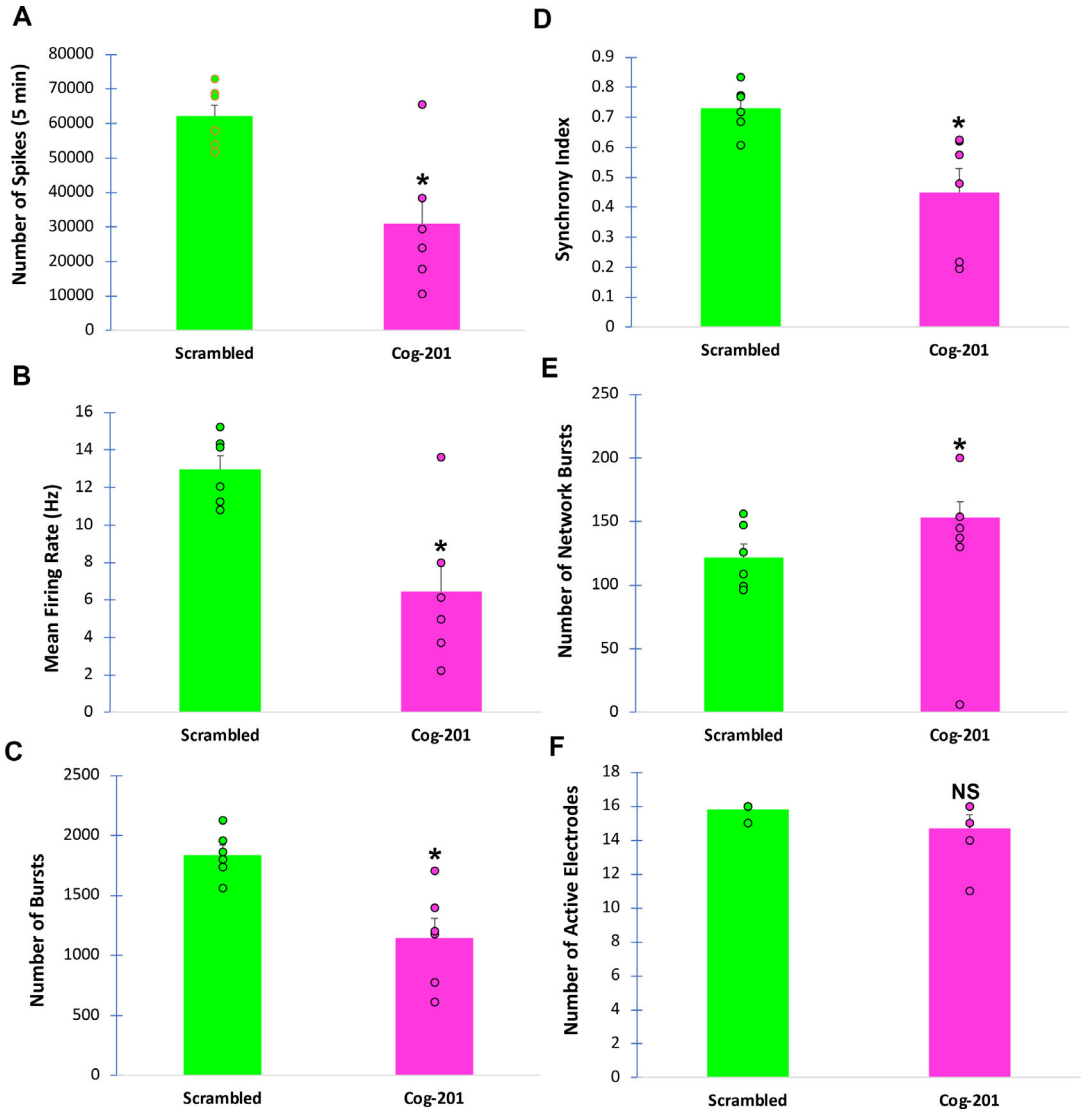


Figure 5. *In vitro* exposure of primary mouse cortical neurons with AAV9-mHTR2A-shRNA leads to a decrease in spontaneous electrical activity. MEA analysis was performed in mouse cortical neurons following treatment with either COG-201 (red bars, labeled "shRNA") or a scrambled shRNA version (black bars, labeled "Scram") at a MOI of 2×10^5. Neurons were treated at day 6 and MEA analyses were performed on day 16. (**A–F**) Quantification of MEA analysis showing the number of spikes over 5 minutes (**A**), mean firing rate (**B**), the number of bursts (**C**), the synchrony index (**D**), which indicates a unitless measure of synchrony between 0 and 1. Values closer to 1 indicate higher synchrony, the number of network bursts defined as a cluster of spikes across all electrodes (**E**), the synchrony index, which indicates a unitless measure of synchrony between 0 and 1 (values closer to 1 indicate higher synchrony), and the number of active electrodes (**F**). Exposure of neurons to 2×10^5 MOI led to a significant decrease in the number of spikes (50% decrease compared to scrambled controls, p-value = .002) (A), the mean firing rate (50% decrease, p-value = .002) (**B**), in the number of bursts (27% decrease, p-value= .015), (**C**), and a decrease in the synchrony index (38% decrease compared to vehicle controls, p-value = .005) (**D**). An increase in the number of network bursts was observed (20% increase, p-value = .04) (**E**). For the number of active electrodes, there was no significant difference between the two groups (**F**), p-value = .09. Data represent N of 6 for all parameters, ±S.E.M.

Conclusions

Our study provides compelling evidence that COG-201 in vivo improves memory compared to vehicle control through a potential combined action of improving retention and lowering anxiety. In vitro, COG-201 led to a significant knockdown of the 5-HT2A receptor at both mRNA and protein levels in primary mouse cortical neurons, as confirmed by real-time qPCR, Western blot analysis, and immunocytochemistry. The reduction in receptor expression correlated with a decrease in neuronal excitability, as indicated by MEA assessments of electrical activity. Specifically, a significant reduction in spikes, mean firing rate, and synchrony index, coupled with an increase in network bursts, implies that COG-201 induces a reduction in overall excitability. However, the increased number of network bursts also suggests a compensatory mechanism within the neural network, that potentially enhances global synchronization. Our interpretation is that the increased network bursts in neurons with reduced 5-HT2A expression at baseline, is indicative of a newly formed neuronal network that has the potential of also increasing long-term potentiation. While this hypothesis can only be confirmed by further experiments, for example, applying electrical stimuli to in vitro neurons, thus mimicking the in vivo effects of the integration of sensory information. Nonetheless, these findings are aligned with previous behavioral observations of reduced anxiety and improved memory following COG-201 administration and underline the potential of COG-201 as an effective therapeutic agent (10). By elucidating some of the functional consequences

of 5-HT2A receptor knockdown, this study provides a critical link between molecular changes and the resultant alterations in neural circuitry that underpin the observed behavioral outcomes. Future research is warranted to explore the precise mechanisms by which COG-201 modulates network behavior and to assess the impact of these findings on therapeutic strategies for disorders including MCI that is characterized by anxiety and memory impairments. However, the current study utilizing mice to assess the efficacy of COG-201 is not without its' limitations. While these animal models are informative, there are significant physiological and genetic differences between rodents and humans that may affect the translatability of these findings to human therapeutics. In this context, the intranasal delivery of shRNA (COG-201) in animal models may not directly translate to humans due to differences in nasal anatomy and absorption efficiency. More research is needed to determine whether this delivery method is viable for human patients. Therefore, conducting studies in larger animal models that are more physiologically similar to humans (e.g., non-human primates) could provide better insights into the potential translational impact of COG-201.

In conclusion, if COG-201 proves effective in humans, it could offer a new treatment option for patients with conditions like MCI, which often involve anxiety and memory problems. This would be particularly beneficial given the limited treatment options currently available.

Declaration of Interests

J.L.M., B.J.L. and D.R. are co-founders of Cognigenics, members of its scientific advisory board, and hold equity in the company. T.T.R. is a part-time consultant serving as Director of Preclinical Research at Cognigenics and in addition to receiving a salary, owns shares of the company's common stock and options for common shares. F.M. is a part-time consultant serving as Chief Science Officer at Cognigenics, Inc., and is a member of its scientific advisory board.

Author Contributions

T.T.R., D.R., and F.M. designed research, analyzed and interpreted data and wrote the manuscript. All other authors reviewed the results and approved the final version of the manuscript. All experiments were carried out independently by contract research organizations.

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Generative Artificial Intelligence Statement

Generative artificial intelligence was not used to write the manuscript, but instead was used as a final tool to improve a human-generated text.

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Rethinking the connection between bipolar disorder and epilepsy from genetic perspectives

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Epilepsy and bipolar disorder (BD) exhibit considerable biochemical and genetic overlap. Our study unveiled a significant genetic correlation ($r_g = 0.154$, $P = 9.24 \times 10^{-6}$) between BD-I and epilepsy, indicating a meaningful causal effect of epilepsy on BD-I ($P = 0.0079$, bxy = 0.1721, SE = 0.0648). Additionally, we identified 1.3k shared genetic variants and 6 significant loci, demonstrating substantial polygenic overlap. Notably, the rs9639379 variant within the SP4 gene exhibited strong associations with both BD-I and epilepsy, implicating SP4 in the etiology of both disorders.

Epilepsy and bipolar disorder (BD) or mania are postulated to share a common biological underpinning. Altered intracellular calcium ion concentration ([Ca^{2+}]) is a consistent biochemical finding in BD and epilepsy (1, 2). Certain antiepileptic drugs act as mood stabilizers by inhibiting calcium currents and are effective in treating patients with epilepsy as well as patients with BD. These findings imply a potential link between mood polarity (particularly mania) and seizures. As both epilepsy and BD have well-described genetic substrates, in this analysis we ascertained shared genetic underpinnings and causal effects and unveiled six independent genomic loci significantly linked to BD and epilepsy.

Utilizing genome-wide association study (GWAS) data from European populations, comprising 26,352 epilepsy cases and 774,517 controls (3), as well as 25,060 BD type I (BD-I) cases and 307,499 controls (4), we observed a significant positive genetic correlation ($r_g = 0.154$, $P = 9.24 \times 10^{-6}$) between BD-I and epilepsy. Furthermore, we indicated a meaningful causal effect of epilepsy on BD-I ($P = 0.0079$, $b_{xy} = 0.1721$, SE = 0.0648).

Our MiXeR analysis identified approximately 7.8K variants influencing BD-I and 3.0K impacting epilepsy, with 1.3K variants implicated in both conditions (Figure 1A). We unveiled six independent genomic loci ($r^2 < 0.2$) significantly linked to BD-I and epilepsy using Conjunctional False Discovery Rate (conjFDR) analysis (conjFDR < 0.05, Figure 1B), among which four loci exhibited consistent allelic effect direction between BD-I and epilepsy, while the remaining two loci showed opposite direction. Moreover, we found that five of the six risk loci showed expression quantitative trait loci associations in cortex tissues or specific cell types ($P < 1.00 \times 10^{-5}$, Supplemental Table S1).

We focused on rs9639379 in the SP4 gene, finding strong associations with both risk of BD-I (odds ratio (OR) = 1.0638, $P = 1.41 \times 10^{-6}$) and epilepsy (OR = 1.0437, $P = 2.31 \times 10^{-5}$) (conjFDR = 1.24×10^{-2}, Figure 1C). The stability of SP4 protein was modulated by neuronal activity, with lithium demonstrating the ability to stabilize SP4 levels, thereby suggesting

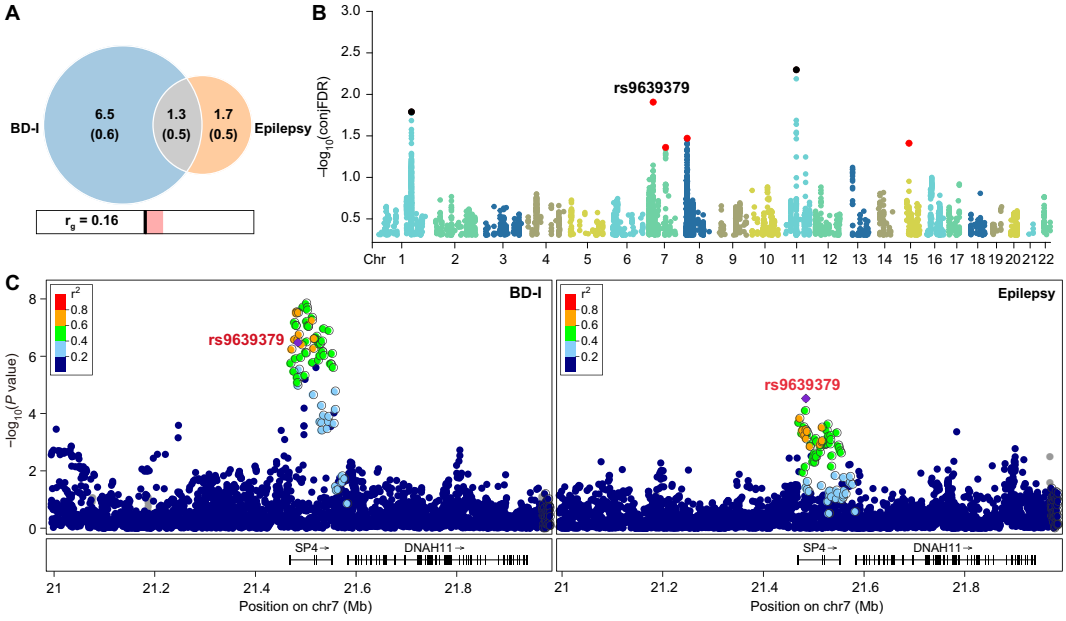

Figure 1. (A) Venn plot shows the number of specific and shared causal variants between BD-I and epilepsy. The genetic correlation of r_g was estimated by Linkage Disequilibrium Score Regression (LDSC). (B) Manhattan plot of conjFDR result. Lead Single Nucleotide Polymorphisms (SNPs) in each independent risk loci with the same direction of allelic effects between BD-I and epilepsy are marked in red, and lead SNPs in each independent risk loci with opposite direction of allelic effects between BD-I and epilepsy are marked in black. (C) LocusZoom plots show the genetic associations with BD-I and epilepsy in the SP4 locus. Physical maps blow the plots depict known genes within the region, and the Linkage Disequilibrium (LD) is defined based on the SNP rs9639379.

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therapeutic benefits in mood disorder management (5). While the direct association between SP4 and epilepsy remains unclear, the involvement of SP4 in the transcriptional regulation of neuronal energy metabolism suggested a plausible link to epileptic seizures (6).

This study provides a novel rethinking of the connection between epilepsy and BD, which is in line with the fact that mood stabilizers are effective in the treatment of both illnesses. Although the relationship between shared risk genes and mood stabilizers is still unclear, their potential involvement in drug-mediated neurobiological mechanisms is worth further investigation. Limitations include the focus on European populations, which may constrain the generalizability of the findings, and the reliance on public GWAS data without sex-specific information restricting us from conducting a gender-based analysis.

Author Contributions

ML oversaw the project, conceived and designed the study and JHH performed the primary analysis and drafted the first version of the manuscript. All authors revised the manuscript critically and approved the final version.

Conflicts of Interest

None declared.

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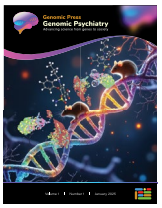

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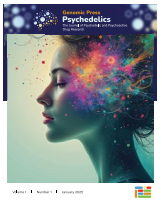

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