Somatic mutations in disorders with disrupted brain connectivity

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Mutations occur during cell division in all somatic lineages. Because neurogenesis persists throughout human life, somatic mutations in the brain arise during development and accumulate with the aging process. The human brain consists of 100 billion neurons that form an extraordinarily intricate network of connections to achieve higher level cognitive functions. Due to this network architecture, perturbed neuronal functions are rarely restricted to a focal area; instead, they are often spread via the neuronal network to affect other connected areas. Although somatic diversity is an evident feature of the brain, the extent to which somatic mutations affect the neuronal structure and function and their contribution to neurological disorders associated with disrupted brain connectivity remain largely unexplored. Notably, recent reports indicate that brain somatic mutations can indeed play a critical role that leads to the structural and functional abnormalities of the brain observed in several neurodevelopmental disorders. Here, I review the extent and significance of brain somatic mutations and provide my perspective regarding these mutations as potential molecular lesions underlying relatively common conditions with disrupted brain connectivity. Moreover, I discuss emerging technical platforms that will facilitate the detection of low-frequency somatic mutations and validate the biological functions of the identified mutations in the context of brain connectivity.

INTRODUCTION

Somatic mutations are genetic variations in somatic cells that are not inherited from a parent and are also not passed on to offspring. Somatic cells in humans are known to acquire two to three mutations per DNA replication and one to nine base-substitution mutations per year in a replication-independent manner. Thus, somatic mutations exist in all somatic lineages and comprise any tissue or organ, and thus, the cells of the human body do not all contain identical genome sequences. Such somatic mutations can be single nucleotide variations (SNVs), copy number variations (CNVs), loss of heterozygosity or large structural variations (SVs). Based on when or where the mutations arise, somatic mutations occur as a mosaic pattern that affects the whole body or they are restricted to a certain tissue or even to a focal area within an organ. For example, somatic mosaic variations caused by early-embryonic mutation events can be distributed across many organs and tissues and are not limited to a given organ or tissue. Genome mosaicism and its implications in human disease have been carefully reviewed in several other papers. In this review, I focus on somatic mutations in the brain and the potentially related neurological disorders.

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mutations in mitochondrial DNA have been reported in the aging brain. Therefore, it is not surprising that somatic mutations in the brain occur during the post-fertilization or post-zygote period and accumulate with aging (Figure 1b).

Regarding neurological disorders, it is well known that somatic mutations cause most brain tumors, which is similar to the occurrence of other human cancers. Other than brain tumors, neurological disease-causing or -associated mutations have been considered inherited or de novo mutations in germ cells, and therefore, they could be detected in any tissue of the affected patient, such as readily accessible blood or saliva. However, damaging mutations in biologically important genes may be incompatible with normal development or the survival of an organism and may not be detected as inherited or de novo mutations in germ cells but might be detected as somatic mutations in a small fraction of the somatic cells. In addition, brain function is more vulnerable to the focal functional or structural damage caused by somatic mutations in comparison to other organs because of the nature of brain connectivity. Nevertheless, the extent and significance of somatic mutations in most brain disorders, including neurodevelopmental, neuro-psychiatric and neurodegenerative disorders, has remained unresolved.

THE IMPLICATION OF SOMATIC MUTATIONS IN DISORDERS WITH DISRUPTED BRAIN CONNECTIVITY

The human brain is an extraordinarily intricate network comprising ~10^{11} neurons with ~10^{15} connections. The connection matrix of the human brain is the so-called ‘connectome’. An important property of an interdependent network, such as the connectome, is that the dysfunction of a node in the network can spread throughout the connections and disrupt the function of the entire network. In the brain, neurons act as nodes, and synaptic and axonal contacts function as connections and conduits for the pathological spread. Therefore, neuronal dysfunctions or abnormalities are not limited to a restricted area but spread to or affect other regions, thereby altering neural connectivity. Indeed, various studies in the human brain using MRI, functional MRI (fMRI), diffusion tensor imaging (DTI) or electroencephalography (EEG) have shown that brain disorders, including neuro-developmental (for example, epilepsy or autism), neuro-psychiatric (for example, schizophrenia (SZ) or depression) and neurodegenerative (for example, Alzheimer’s disease) disorders are associated with the altered structure or function of the focal regions and the disrupted neural connectivity in the brain. Consistent with these observations, such conditions are often understood as disorders with disrupted brain connectivity.

What is the genetic etiology of such brain disorders? These conditions are often familial, and twin studies have indicated that their concordance rates range from 20 to 90% (20–75% for epilepsy; 70–90% for autism; ~80% for SZ; 40% for major depression and 74% for Alzheimer’s disease). These findings suggest that genetic factors play a major role in the development of these disorders. Inherited or de novo genetic variations, including SNVs and CNVs, are considered the most likely genetic etiology of disorders with disrupted brain connectivity. Although such concordance rates imply that heritable variations substantially contribute to these conditions, these diseases frequently occur as isolated cases. As a possible explanation for this, de novo mutations in germ cells have also been considered to account for the genetic etiologies of most sporadic cases that are not sufficiently explained by inherited mutations. Indeed, recent large-scale genomic studies using whole-exome or whole-genome sequencing in peripheral tissue samples (for example, blood) from patients and their family members showed that the candidate genes harboring recurrent de novo mutations are implicated in epilepsy, autism and SZ. However, the identified de novo mutations explain only a
limited portion of the sporadic cases. For example, a recent large-scale exome sequencing of peripheral samples from 264 patients–parent trios with epileptic encephalopathy identified genetic causes in ~7% of the patients in the cohort. In autism, recent whole exome sequencing (WES) studies suggest that de novo SNVs and CNVs contribute to the risk of autism in ~30% of the sporadic cases in simplex families and that no single gene accounts for >1% of autism cases. In SZ, de novo mutations appear to contribute to ~25% of all sporadic cases. However, the genetic contribution to most of the sporadic cases remains unanswered. Regarding this point, cancer genomics may offer some instructive insight to explain the genetic contribution to the sporadic form of brain network disorders. Although cancers have a substantial heritability with several defined mutations at the level of the germ cells, most of the mutations underlying cancer arise somatically in affected

Figure 2 Low-level somatic mutations in a focal brain region cause the synchronized electrical discharge of the entire brain network. (a) Post-op MRI and pathology from focal cortical dysplasia II (FCDII) with intractable epilepsy show the resected brain region (white arrow) and the scattered dysplastic neurons (black arrow) with the mTOR-activating somatic mutation (p.Leu2427Pro). Within the resected brain tissue, deep WES detected 6.9% mutated alleles. Scale bar, 50 μm. (b) In utero electroporation of the mTOR p.Leu2427Pro mutant plasmid into the focal area of the embryonic brain (E14) followed by monitoring of the mTOR mutant expression using a GFP reporter. Imaging of the GFP signal in the coronal sections of an electroporated mouse brain at ~3 months of age shows a small fraction of GFP-positive cells expressing the mutated mTOR, accounting for 1.30% of all the DAPI-positive cells in the left cerebral cortex. Scale bar, 500 μm. (c) The in utero electroporated mouse exhibits a synchronized epileptic discharge with behavioral seizures by video-electroencephalograph (EEG) monitoring. LF, left frontal electrode; LT, left temporal electrode; RF, right frontal electrode; RT, right temporal electrode. (d) The schematic figure shows how somatic mutations in a focal region can affect the function of the entire brain via the brain connectivity. (a, c) are adopted from ref. 48. GFP, green fluorescent protein; MRI, magnetic resonance imaging.
tissues or cell types, and these somatic mutations can explain the genetic contribution to sporadic cancers. Among the >20,000 protein-coding genes, cancer genomic studies, to date, have identified ~140 core genes that are recurrently mutated and drive tumorigenesis in affected but not in unaffected tissues. The occurrence of somatic mutations demonstrated in cancer genomics may be applicable to and testable in sporadic cases with disrupted brain connectivity. However, due to the difficulty associated with accessing brain tissues and the limitations of the sequencing technology for detecting low-level somatic mutations without cell-proliferative effects (as discussed later in this review), the extent of the brain somatic mutations in these diseases has remained largely unexplored. Recently, the notion of brain somatic mutations has received great attention as the underlying pathogenesis in focal epilepsy. Herein, I first review the current evidence for the pathogenic roles of brain somatic mutations in epilepsy. Subsequently, I provide my perspective regarding brain somatic mutations as a potential molecular lesion underlying other conditions of disrupted brain connectivity, such as autism spectrum disorder (ASD), SZ, major depression disorder and Alzheimer’s disease.

Epilepsy
Epilepsy is a typical brain disorder with neural network dysfunction, including excessive and synchronous electrical activity throughout the brain. Approximately 60% of the people with epilepsy have focal seizures that originate in just one part of the brain. These seizures often spread to the other side of brain or the entire brain via the neural connection and contribute largely to the development of chronic epilepsy. Approximately 20–30% of epilepsy cases are medically refractory and such intractable epilepsies often require epilepsy surgery to resect the suspected origin of the seizures as an alternative treatment, which allows access to the affected brain tissue for the testing of brain somatic mutations. In particular, for a long time it was thought that somatic mutations might play a role in intractable epilepsy combined with focal cortical malformations because most of these diseases occur sporadically without a family history and sometimes show discordance in monozygotic twins. For a rare condition, called hemimegalencephaly (HME), which presents an enlargement of one side of the brain and is associated with intractable focal epilepsy, we and others provide evidence for somatic mutations in the PI3K–AKT–mTOR pathway genes and thereby suggest that brain somatic mutations might be important for other neurodevelopmental disorders. Somatic mutations accounted for up to 30% of the studied HME patients, and the frequency of the mutated allele ranged from 8 to 39% in the enlarged brain tissues. Recently, brain somatic mutations in MTOR and its related genes were identified in up to 25% of the patients studied with focal cortical dysplasia, which is more common and accounts for up to 50% of the children undergoing epilepsy surgery. Notably, brain MRIs from patients carrying somatic mutations were frequently reported to be normal, which thereby implies an extremely low level of mutational burden in the affected areas. Indeed, the frequency of the mutated allele was reported to be as low as ~1% in the resected brain tissues, but the mutated allele was not detected in the peripheral samples, such as the blood or saliva. More surprisingly, such a small fraction of single nucleotide mutation-carrying neurons were sufficient to cause intractable epilepsy via the neural network in a mouse model (Figures 2b and c). This finding provides direct evidence that the low-level brain somatic mutations found in patients indeed cause the brain network disorder by disrupting the entire brain function as a whole. Although these recent studies revealed the extent of the brain somatic mutations in only a few examples of intractable epilepsies that permitted access to the surgically resected brain tissues, these findings imply that somatic mutations that alter the excitability in the affected brain regions might be responsible for a substantial proportion of the epilepsy cases that remain unexplained by genomic studies in the peripheral samples.

Autism spectrum disorder
ASDs are associated with disrupted brain connectivity. However, the genetic etiology that results in the disruption of brain connectivity in ASD remains poorly understood. Recent ASD family-based genomic studies show that de novo genetic variations occurring at the germline level are significantly associated with ASDs, but specific causes, including mutations in the synapse proteins, were identified in only a modest fraction of the individuals with ASDs. Moreover, a recent study showed that most of the observed de novo mutation events in the germline have limited effects on ASD and that those that do confer a risk are not necessarily sufficient for disease, which thereby implies the possibility of a genetic cause of ASD that may not be detected at the germline level. Many functional and structural MRIs studies in ASD show altered activities and increased cortical thickness in specific brain regions, including the prefrontal cortex that mediates autistic symptoms. In addition, neuropathological findings from the post-mortem brains of autism patients indicated the presence of focal abnormalities in the prefrontal cortex, including a patched cortical dyslamination and dysplastic changes in the neurons. Interestingly, the prefrontal cortex in humans is known to be the major target area of the new neurons generated from robust postnatal neurogenesis in the subventricular zone. The burden of the somatic mutations can accumulate during neuronal cell proliferation. Therefore, it is worth testing the hypothesis that somatic mutations in the affected brain regions, such as the prefrontal cortex, participate in the pathogenesis of ASD.

SZ and major depressive disorder (MDD)
Can brain somatic mutations be a pathogenic factor in neuropsychiatric disorders such as SZ and MDD? Although the contribution of brain region-specific somatic mutations in mitochondrial DNA has been explored in neuropsychiatric conditions of disrupted brain connectivity, such as autism spectrum disorder (ASD), SZ, major depression disorder and Alzheimer’s disease.
disorders, investigators have recently increasingly recognized the potential role of brain somatic mutations in the genomic DNA. The high discordance rates (~40–60%) in monozygotic twins have raised the possibility that de novo mutations might be implicated in these mental disorders. Consistent with these findings, recent large-scale genomic studies identified the association of SZ and MDD with several common or rare de novo genetic variations. However, these findings are insufficient to explain most of the cases of sporadic SZ and MDD. Although numerous efforts, such as the identification of disrupted synaptic networks caused by multiple de novo mutated genes or epigenetic changes, have been made to find the other level of genetic contribution, this unexplained genetic contribution gives rise to the possibility that the genetic variations implicated in SZ and MDD may not be easily detected in peripheral samples such as blood and saliva but rather may be localized to the affected brain areas. Dysfunction in the prefrontal cortex, which is critical for converting the external stimuli into a response, is frequently implicated in SZ and MDD. Interestingly, it was recently reported that the somatic copy number of L1 retrotransposition in the prefrontal cortex increases in SZ and MDD patients, and the brain-specific L1 insertion is preferentially localized to synapse-related genes in SZ. In addition, somatic deletions are reported in the prefrontal cortex of SZ patients, as well as unaffected controls. To date, only a few have investigated brain somatic mutations in neuropsychiatric disorders, and no studies have found SZ- and MDD-associated brain-specific SNVs or the pathogenic causality of the brain somatic mutations observed in SZ or MDD yet. Therefore, additional systematic studies are needed to test the hypothesis regarding the pathogenic role of brain somatic mutations in neuropsychiatric disorders.

Alzheimer’s disease

Investigators have focused on identifying the molecular genetic causes of Alzheimer’s disease (AD) for more than a century. Indeed, these efforts enabled the identification of the disease-causing mutations in the APP or Presenilin 1 or 2 genes in early-onset familial AD and the disease-associated mutations in the APOE4 or TREM2 gene in late-onset AD. However, these genetic variations were absent in the peripheral genomic DNA collected from most cases of sporadic AD (sAD). Most sAD has the characteristics of a sporadic occurrence, a focal onset of pathology and a progressive spread of the protein aggregates. These features have raised the hypothesis that brain somatic mutations contribute to neurodegenerative disorders including sAD. Neurofibrillary tangles parallel the neuronal loss and progression of cognitive decline. Interestingly, neurofibrillary tangles first appear in the hippocampal area (for example, entorhinal cortex) and spread to the other brain regions, including the neocortex, and thereby, correlate with the symptomatic progression. Somatic mutations accumulate with aging, and their occurrence increases in proliferating cells. Considering that hippocampal neurogenesis continues during the aging process and that one-third of the hippocampal neurons are subject to change over a life-time, it is plausible to hypothesize that the accumulation of somatic mutations in the hippocampal regions may contribute to the production or progression of neurofibrillary tangles, which might play a role in the potential pathogenesis of AD. Indeed, it was recently reported that brain somatic mutations with a low allele frequency are present in the entorhinal cortex or hippocampal genomic DNA from patients with sAD. However, these studies still lack the direct evidence that brain somatic mutations contribute to or are associated with the pathogenesis of sAD. Therefore, further studies with larger samples or more systematic deep sequencing at the genome-wide level will be necessary to test the hypothesis that brain somatic mutations are a genetic cause of sAD.

DETECTING BRAIN SOMATIC MUTATIONS WITH A LOW ALLELE FREQUENCY

Unlike somatic mutations, which have highly proliferative effects in human cancers, brain somatic mutations in non-cancerous neurological disorders are likely to have no or fewer proliferative effects. Consequently, mutation-carrying cells can be surrounded by a large number of normal cells, and the allele frequency of the somatic mutations in the affected brain regions can be much lower than the typical mutational burden found in tumor samples. For example, recent studies using ultra-deep sequencing showed that somatic mutations in the affected brain of intractable focal epilepsy or sAD patients were detected in as low as 1% of the mutated allele. In particular, the low level of the brain somatic mutations found in intractable focal epilepsy was sufficient to cause a spontaneous seizure (Figures 2b and c). Therefore, it is important to sensitively and accurately detect the brain somatic mutations that occur at a low allele frequency. To achieve this goal, several criteria should be considered: (1) the use of different sequencing platforms with a high read depth; (2) single-cell sequencing or an enrichment of the mutated cells; and (3) the selection of the suitable variant callers.

The use of different sequencing platforms with a high read depth

Using traditional Sanger sequencing, which is generally considered the gold standard to confirm SNVs, it is difficult to detect <10–20% of the low level mutations. To detect such low-level mutations, high-coverage sequencing that quantifies the level of the mutations is necessary. A read depth of >1000 x is known to detect an allele frequency of 1% with >90% probability. Even with such a high read depth, unexpected sequencing artifacts or erroneous calls can arise from the sample, and library preparation or sequencing and imaging specific to the sequencing platforms can often mimic true somatic mutations with a low frequency. For example, hybrid capture and PCR-based amplicon sequencing may have unique artifacts arising during the library preparation. In addition, according to the type of sequencing platform used, including Illumina, Ion Torrent, PacBio and digital PCR, the sequencing error rate ranges from almost 0 to ~4% with
different types of errors, such as substitution, indel and deletion. Therefore, an increased sequencing read depth and various experimental replications, such as technical, biological and cross-platform replications have been proposed as tools to minimize such false positive or erroneous variants. Indeed, technical and biological replications, which are commonly performed in molecular biology experiments, as well as cross-platform replications with a high read depth, reduce the false variants and detect the true variants when the mutated alleles are present at 1%. Nevertheless, further studies that optimize the read depth and experimental replications with different platforms will be necessary to provide an accurate and cost-effective method for detecting somatic mutations with a low allele frequency.

### Single cell sequencing or enrichment of the mutated cells
The sequencing technologies with a high read depth described above utilize pooled genomic DNA isolated from brain tissues containing many distinct cell types. By contrast, single cell sequencing reads the genome of individual cells, which thereby pinpoints specific cells or cell types that contain somatic mutations in a given brain tissue. To do this, single cells should first be isolated from brain tissues using various techniques, such as fluorescence-activated cell sorting, laser capture microdissection (LCM) and microfluidic devices, which have unique technical advantages and disadvantages (for example, unbiased vs biased sampling or high- vs low-throughput). The single-cell genome (for example, ~6 pg DNA per a cell) is then amplified to obtain sufficient amounts of DNA for next-generation sequencing (for example, 250–500 ng DNA for whole genome or exome sequencing). The use of such single-cell sequencing technologies has revealed that rare somatic CNVs and L1 retrotransposition events occur in the normal and diseased human brain. However, single-cell genome sequencing methods, especially for the detection of SNVs, are challenged by extensive technical errors and poor physical coverage data arising due to the nature of the whole genome amplification process. Many ongoing investigations are currently focused on overcoming these technical challenges. Alternatively, if researchers are able to specify or label the neuronal cells that potentially carry the mutations, the enrichment of these cells using single-cell isolation methods could be followed by sequencing without whole genome amplification, which might lead to the identification of brain somatic mutations with a low allele frequency. For example, a recent study of brain somatic mutations in epilepsy showed that even Sanger sequencing in a pool of 20–100 dysplastic neurons enriched by LCM was able to validate the low-level somatic mutations that were originally presented as ~5% of the mutation burden in the affected brain tissues.

### The selection of suitable variant callers
Most of the variant callers used to detect somatic mutations from whole genome or exome data were developed mainly in the field of cancer genomics. In general, these callers are designed to detect somatic mutations with a high frequency by jointly analyzing the paired diseased-normal samples from each patient. Recently, due to the challenges associated with tumor heterogeneity, several callers, such as MuTect, Strelka and Virmid, have been developed to detect low-level somatic mutations in impure tissue samples with a high sensitivity and specificity. By adopting these variant callers in recent studies of intractable focal epilepsy, brain somatic mutations present as 1–10% of the mutation burden could be identified from deep whole exome sequencing data in matched brain–blood (or saliva) tissues. However, the sensitivity of these callers significantly declines in samples with <8% of the mutation burden, and they only detect SNVs or small indels in paired samples and not low-level CNVs and SVs, which might be implicated in disorders with disrupted brain connectivity. In addition, considering the difficulty related to gaining access to matched brain-peripheral samples from patients with the somatic mosaicism that is present at a low level in the brain and other peripheral tissues, the ideal somatic variant callers should be able to detect low-level SNVs, as well as CNVs and SVs in unmatched or matched tissue samples. Although a few recently developed algorithms address some of these issues, such as the low-frequency SNVs or deletions in the unmatched samples, further studies are needed to develop more ideal variant callers.

### VERIFYING THE BIOLOGICAL FUNCTIONS OF THE SOMATIC MUTATIONS IN THE CONTEXT OF BRAIN CONNECTIVITY
Recent advances in sequencing technologies and bioinformatics analysis tools have greatly improved the comprehensive catalogue of low-frequency somatic mutations that are present in genetically heterogeneous tissues. However, to verify the biological and pathological effects of such variants, laboratory experiments at the level of the cell or organism are essential. Although it has been difficult to design experiments that recapitulate the somatic alterations of the eukaryotic genome, sequence-specific programmable nucleases provide a new opportunity for the targeted modification of the DNA sequence itself. In particular, the RNA-guided CRISPR-associated Cas9 nuclease enables a convenient system for achieving targeted somatic mutagenesis in vitro, as well as in vivo. The CRISPR–Cas9 system can be customized for the specific purpose of somatic genomic editing. In this system, double-strand break generation by the introduction of Cas9 and single-guide (sg) RNAs targeting protein-coding regions induce loss-of-function frameshift indel mutations via non-homologous end-joining. The addition of donor DNA or single-strand deoxyoligonucleotides that contain mutated sequence introduces precise mutations, including gain-of-function one via homology-directed repair. For example, the biological consequences of somatic loss-of-function alterations at the cellular level can be screened via high-throughput functional genomics using the CRISPR–Cas9 systems with lentiviral single-guide RNA libraries. The somatic loss-of-function or gain-of-function mutations found
in patients with lung and liver cancers were successfully recapitulated by CRISPR–Cas9-mediated genome editing in mouse models. In particular, to understand the biological effects of somatic mutations at the level of brain connectivity, in vivo genome editing in the brain may be critical. In agreement with this notion, recent studies show that the delivery of Cas9/sgRNA vectors using in utero electroporation or adeno-associated virus (AAV) was able to edit the genome of neurons and phenocopy brain tumours or Rett-syndrome with learning and memory deficits in the mouse brain. To date, none of the brain somatic mutations identified in patients with disrupted neural connectivity, such as intractable focal epilepsy, have been modeled by somatic genome editing, but modeling of the brain somatic mutations found in patients via CRISPR–Cas9-mediated genome editing appears to be achievable in the near future.

In addition to advances in genome editing technology, a wide range of experimental tools in neuroimaging and electrophysiology have been developed to investigate the structure and function of brain connectivity at many levels, from the whole brain to neurons and to individual synapses. DTI delineates white matter tracts to investigate structural connectivity; whereas, fMRI and EEG examine the functional connectivity or network activity at the whole brain level. In vivo two-photon imaging using viral tracers or calcium/voltage-sensitive indicators, multi-electrode recordings of local field potentials and optogenetic approaches permit the examination of structural and functional changes in the neuronal interconnections at the local circuit level. Electron-microscopy with imaging reconstruction allows the visualization of neuronal connections at the level of the synapse. Together with in vivo somatic genome editing, these experimental tools will aid in verifying the biological or pathological effects of somatic mutations on the structure and function of brain connectivity. Furthermore, the successful generation of disease models with brain somatic mutations will facilitate the validation of therapeutic targets genetically identified in each patient, which will thereby pave the way toward the realization of precision medicine in brain disorders in the near future.

CONCLUSIONS AND FUTURE DIRECTIONS

Our understanding of the extent and roles of somatic mutations in brain disorders is limited due to the difficulties associated with detecting low-level somatic mutations in surgical or postmortem brain tissues and verifying their functional consequences in the context of the neural network. However, recent advances in technology, for example, single-cell sequencing or deep sequencing with proper bioinformatics analysis, somatic genome editing and experimental tools for investigating brain connectivity, will enable a better understanding of (1) the prevalence of somatic mutations in disorders with disrupted brain connectivity; (2) the biological and pathological effects of somatic mutations on the structure and function of brain connectivity; and (3) the physiological roles of somatic mutations during brain development or aging, as well as provide improved guidance for (4) the development of precision medicine for brain disorders by discovering the genetically identified targets similar to those identified in cancer medicine. We are confident that studies of brain somatic mutations will substantially advance our knowledge of the genetics, pathogenesis and therapeutics of common but unexplained disorders with disrupted brain connectivity.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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