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Next generation sequencing in genetic diagnostics[1)]

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Abstract

The introduction of next-generation sequencing technologies in human genetic diagnostics is a challenge to many of its aspects. It is mainly positive, even revolutionary, which will be discussed as well as its critical aspects. It used to take several months or years to complete genetic testing. This already belongs to the past. As sequencing technologies are progressing at an extremely high speed, Germany recently introduced the new Gen Diagnostics Law in February 2010. Four common diseases with genetic contribution (dementia, Parkinson's syndrome, epilepsy, and hereditary eye diseases) will be used to exemplify the latest development of human genetic testing.

Keywords: diagnostic panels; next-generation sequencing; SOLiD 4 technology.

Introduction

Genetic or molecular genetic diagnostics refers to the study of individual genes that, when changed, most probably lead to the manifestation of a disease or are the cause of a disease. A genetic cause must be considered especially when the afflicted persons are of young age. The sum total of hereditary material in humans is stated to be approximately 3 billion DNA base pairs and codes for 20 to 30,000 genes. A change in the hereditary material may be transmitted from generation to generation or it may arise "de novo". A distinction is made between changes in germ cells, which are hereditary, and somatic changes, e.g., tumor cells. In this paper, the term "mutation" (change in the hereditary material) is replaced with "variation", in order to do better justice to the different types of variations. There are pathogenic variants that are certain to cause disease, but there also are variants whose significance is not explicitly clear. These include the "probably pathogenic", the "probably non-pathogenic", the "probably benign", the "certainly benign" and finally an ever increasing group of variants of uncertain significance (VUS).

The first large catalog of genes that, when changed, are associated with a disease was compiled into a database named OMIM (Online Inheritance In Man) by the American human geneticist Viktor McKusick (1921–2008). As a consequence, he is seen as the founder of medical genetics. The data base grows larger every day and is administered by the Johns Hopkins University in Baltimore, USA. So far variations in 10% of all human genes could be associated with known phenotypes. That is a comparatively large number and yet does not explain the majority of genetic variability in man.

Genetic diagnostics is useful if it brings about consequences from a therapeutic or prophylactic perspective or eases the burden on the individual concerned and the family. The right not to know exists at any time and this should be emphasized during genetic counseling before as well as after testing. What follows are examples from the day-to-day practice that illustrate why human genetics rightly sees technological progress as a quantum leap for its own discipline, for the patient and for the health care system.

From exomes to diagnostic panels in human genetics

By means of the sequencing machines available today, high-throughput sequencing permits the simultaneous sequencing of 100 billion basepairs within one week, i.e., the haploid human genome with an average coverage of 30. Because of higher accuracy and higher coverage and for reasons of better understanding of variation it has advantages to focus on the coding regions of the human genome. This is called exome-sequencing; the exome is the totality of all coding regions in the genome. Sequencing an exome is useful if (i) there are strong indications for a genetic disease, (ii) all known genes associated with the disease at issue have been ruled out and (iii) this approach is applied and evaluated by experts in the particular field within the scope of a research project.

In contrast, a diagnostic panel is the targeted simultaneous screening of a list of known genes that have already been described as the cause of a specific disease. Up to now genetic

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diagnostics in such cases was not only very time-consuming, but was frequently not performed because of high costs.

A diagnostic panel clearly differs from the purely scientific and explorative approach of exome-sequencing. A diagnostic panel is ordered by the physician. Only the genes whose connection with the disease has been established are investigated. Finally, a finding is made and forwarded to the physician. This finding contains detailed information about detected variants that are validated through Sanger sequencing and interpreted in relation to the disease at issue.

Using a diagnostic panel on a newest generation high-throughput sequencer makes sense only if several or very large genes can be considered for the disease. Some examples are hereditary tumor diseases with the known breast cancer-causing genes *BRCA1* and *BRCA2*, cardiomyopathies, cardiac dysrhythmias, familial hypercholesterolemias, mental retardation, epilepsies, hereditary ophthalmic diseases and neurodegenerative diseases. Collaboration with clinical experts is indispensable when compiling the gene list of a panel and also later when interpreting the data. Since gene lists can be from two to more than one hundred genes in length, advance clinical delimitation is important. After the patient has given his written consent his DNA is enriched and sequenced with all genes contained in the gene list. The clinical expression of the disease then determines the sequence of the genes to be evaluated.

Before a diagnostic panel can be offered commercially for diagnostic purposes it must first be validated. Within the framework of such validation patients whose variations are already known are "post"-sequenced on the new panel. This requires that 100% of the known variations are found by means of the panel. Furthermore, the enrichment of the genomic regions must be efficient, specific and reproducible. Only then can a diagnostic panel be used for a clinical objective.

But is it then actually useful? We shall closer examine this question with the help of three examples. Detlef Boehm (CeGaT GmbH), a pioneer in establishing new methods in human genetic diagnostics [2] and the Practice for Human Genetics in Tübingen together with clinical partners and with Applied Biosystems/Life Technologies have put special emphasis on hereditary ophthalmic diseases, epilepsies and neurodegenerative diseases when developing diagnostic panels. The aim here is to clarify the genetic cause in affected families and thereby to (i) secure a clinical diagnosis, (ii) be able to offer a targeted examination of other family members, (iii) make possible an early therapeutic intervention, (iv) provide a prognostic assessment of the course of the disease and (v) provide the basis for new therapeutic methods in the long-term.

Diagnostic panel and hereditary ophthalmic diseases

Worldwide hereditary ophthalmic diseases affect several million people. The disease usually starts during adolescence and initially often appears as night blindness. Thereafter the loss of sight as a rule progresses slowly and can take decades. The loss of sight may occur in isolation or in connection with other symptoms. There is no treatment in most cases. As of now a total of more than 180 genes have been described that, when defective, can cause hereditary ophthalmic disease [1]. The description of this list of genes, which has grown so markedly in just the last few years, has provided the first substantial clues for understanding the pathogenesis of the disease and thus has contributed considerably to understanding the disease itself. The gene or its product is not directly sufficient for developing any new medication, but it is the central starting point or point of action of a therapy.

At present approx. 50% of all familial ophthalmic diseases are clarified genetically. Diagnosing frequently takes several years and, due to the investigation of many large genes, is very expensive. Such considerations as well as the need to clarify the other 50% have resulted in the transfer of hereditary ophthalmic disease diagnostics to the high-throughput sequencing arena. The Retina-All-Panel was developed in collaboration with the ophthalmic geneticists Prof. Wolfgang Berger and Dr. John Neidhardt (University of Zürich, Institute for Medical Genetics) and Prof. Bernd Wissinger and Dr. Susanne Kohl (University of Tübingen, Molecular Genetic Laboratory of the Eye Clinic). It contains all presently known genes with a connection to hereditary ophthalmic disease. Sequencing this large panel of genes takes about one to two weeks. Detected variants are issued in a list, classified according to their importance (pathogenic, benign or VUS) and then verified via the Sanger method. Finally, the results are evaluated, summarized and interpreted. Altogether the diagnosis at this time requires no more than two to three months.

A simultaneous study of more than 180 genes does not make sense from a clinical point of view. The list of genes can be divided into smaller groups for the ophthalmic geneticist and clinician. This allows for the simultaneous study of 26 genes in a case of autosomal dominant pigmentary retinopathy, of 28 genes in the case of autosomal recessive pigmentary retinopathy, of 10 genes in the case of Usher's syndrome, 11 genes in the case of congenital stationary blindness, 14, 9 or 5 genes, respectively in cases of Bardet-Biedl, Joubert or Refsum syndrome. A current and comprehensive article on this subject provides more information on the subdivision of ophthalmic diseases and a description of the associated genes [1].

Research in the field of hereditary ophthalmic diseases also gains substantially from the Retina-All-Panel. With the use of the Retina-All-Panel many more patients carrying a pathogenic variant in one of the candidate genes are being identified. Hence, the pool of individuals affected by a certain variant is growing worldwide and the clinical observation of the course of the disease in these patients allows better classification and prognosis assessment in additional patients with the same variant. In the future, it would also be useful from a therapeutic perspective to group patients based on their genetic background when testing new medications, in order to better interpret positive effects as well as side effects.

Next generation sequencing also has an additional scientific aspect. From a purely technological standpoint it makes no difference whether two, one hundred or several thousand patient genes are enriched and sequenced. With a clear diagnostic objective this would be senseless, since data are generated whose required evaluation and validation is difficult and very time-consuming. The situation is different with a family where no genetic cause can be found in the known genes. This presents a possibility that genes as yet not associated with the disease could be studied for the first time and named as the new cause for the disease. In the case of ophthalmic diseases candidate genes are genes that have an important function in the eye but that have not yet been studied in the patients. They are candidates for the cause of ophthalmic disease. This is where the enormous potential of next generation sequencing, that would further advance the knowledge about the causes of hereditary ophthalmic diseases, is to be found.

Diagnostic panel and epilepsy

Epilepsies affect 1% to 3% of the population in the course of a lifetime. The various expressions of the disease are differentiated depending on age and the form of progression. A genetic cause is probable if a symptomatic cause from brain damage, from a tumor, an infection or a metabolic disturbance can be ruled out. Large families with frequently occurring epileptic disease have contributed to the identification of genes and crucially also to the clarification of the pathogenesis of the disease. Identified genes include above all voltage-dependent ion channels and the receptors of neurotransmitters. Conceptually a change in the neural transmission of nerve cell to nerve cell, caused by defective ion channels or neurotransmitter receptors, fits the cause of a convulsive disorder. Specific therapies, e.g., the targeted attack of a medication on a defective sodium channel (valproic acid, carbamazepine, oxcarbazepine and phenytoin) or on a defective receptor (GABA receptor, phenobarbital), provide efficient treatment of a patient. The main goal therefore is the molecular genetic discovery of the cause in as many cases as possible, in order to create a specific individual treatment of the disease. Numerous genes that have been described as the cause for the various forms of familial epilepsy also are possible candidate genes for non-familial cases. Since ion channel genes in particular represent especially large genes, the development of a diagnostics panel for high throughput screening seemed the obvious choice. An epilepsy panel for clinical use was developed by Dr. Johannes Lemke, University of Bern together with the groups around Prof. Holger Lerche and Prof. Ingeborg Krägeloh-Mann (Neurologic and Pediatric University Clinic, Tübingen) in collaboration with CeGaT GmbH. At present the list of genes for purely diagnostic objectives consists of 55 genes and has been clinically subdivided into generalized/myoclonic epilepsies including febrile seizures and absences (a total of 24 genes), epileptic encephalopathies (a total of 8 genes) and syndromal diseases with epilepsy (a total of 23 genes). The panel contains another 450 candidate genes that are being studied for research purposes.

Diagnostics panel and Parkinson's disease and dementia

Parkinson's disease together with Alzheimer's disease is one of the most frequently occurring neurodegenerative diseases worldwide. Both diseases most often occur sporadically and as a rule manifest themselves in individuals above 65 years of age. With a steadily rising life expectancy Parkinson's and Alzheimer's represent one of the greatest medical and socio-economic challenges of the future. In most cases the cause of the death of nerve cells remains a mystery. At the present time it is impossible to predict whether and when an individual will be affected by the breakdown of nerve cells. Once symptoms occur, however, the majority of affected nerve cells have already died. Hence, current treatment concepts have little or no effect, since the time of intervention is years too late. A molecular genetic examination does not immediately offer itself. Why would an individual want to know whether he or she has a predisposition for a neurodegenerative disease as long as no therapies are available? Genetic causes for both Parkinson's and Alzheimer's have been described for slightly more than 10 years. These genetic studies have made important contributions to understanding the breakdown of nerve cells. Intensive research is being done on the gene products in order to speed up new and innovative therapy concepts. The identification of mutated genes has for the first time made it possible to describe biomarkers, in this case "genetic biomarkers", that can predict the occurrence of the disease at a future point in time with a high degree of probability. This allows us to define a group of individuals who could get access to therapies decades before any manifestation of disease. Even though at present such therapies are not yet available, it seems safe to say that the changed gene products will most probably represent the points of action for the therapies of the future.

The key to the changed genes were families in which dementia or Parkinson's disease occurred with great frequency. In the case of Parkinson's disease 16 gene locations for familial autosomal recessive and dominant forms have so far been described in the hereditary [3, 4]. Since only a small part of approx. 5% of familial cases can currently be genetically clarified, the expectation is that the list of genes causing Parkinson's disease will grow. The greater part of the knowledge we possess today about the pathogenesis of Parkinson's disease derives from those genes that have been described in connection with the disease. The gene therefore is the first clue concerning the location of the malfunction within the diseased cell. Like pieces of a puzzle other genes will add to the complex picture of neurodegenerative diseases and – it is hoped – allow a further crucial step in our understanding of the disease in the near future.

The current Parkinson's dementia panel is studying 16 genes in the case of Parkinson's disease, 19 genes in the case of dementia. The panel was created together with Prof. Thomas Gasser (Neurology and Hertie-Institute for Clinical Brain Research, University of Tübingen). The list of genes will grow rapidly, not least due to the possibility of examining families affected by the disease for changes in the total

genome through a method free of hypotheses and within the scope of a research objective.

As with a purely diagnostic objective and within the framework of the genetic diagnostics law the individual seeking advice should also be informed concerning the research objective. Most particularly this includes information on the possible handling of incidental findings with relevance for all family members, the possible destruction of the probe after the examination is completed, the anonymization and use of the probe for further studies, and the right not to know at any point in time.

Outlook and open questions

From the perspective of human genetics the quick and cost efficient sequencing of several thousand human genes within a few days is revolutionary. Also revolutionary is the prospect of personalized medicine in which each single human and each single tumor can be sequenced. It is hoped that with this knowledge diseases might someday be treated individually, i.e., much more targeted than today. Each human is unique, each tumor is unique, each disease with its individual genetic background is unique. Medications therefore have different effects in different people. A changed gene is an essential key for understanding a disease. Even if individualized therapy, e.g., with neurodegenerative diseases, lies in the distant future, the foundation for the therapies of the future is being laid now. For some time tumor genetics has made targeted therapy partially possible, other diseases have followed and more will follow. With all the euphoria that genetics has been experiencing for some time it must be remembered that the results of high-throughput sequencing bring up questions that cannot be answered at this time. This includes the identification of as yet unknown variants in the genome of individuals, variants of uncertain significance (VUS). Add to this that, in spite of high-throughput sequencing, in many cases the cause of a disease cannot be found, be it that no genetic cause exists or that the cause lies in the non-examined regions of the genome, or that it is the synergy of several changed genes with the environment that results in the disease, or that changed gene products (RNA or proteins) are the actual cause of the disease.

High-throughput sequencing or “deep sequencing” is a screening method. Hereditary material is illuminated and it can happen that variations are found that are characterized as incidental findings. Dealing with incidental findings, particularly if they have serious consequences for the patient, is a considerable challenge for the physician and the individual seeking counseling. As with the findings of variants of uncertain significance the patient must be informed about this issue in advance. The result of a genetic examination should be conveyed within the framework of a consultation. The law on gene diagnostics, in force since February 2010, established guidelines for the performance of genetic diagnostics that can also be directly applied to high-throughput diagnostics.

It is hoped that high-throughput diagnostics will arrive in many laboratories and as a consequence will not only contribute to markedly higher clarification quotas of genetically caused diseases, but also will establish genetic diagnostics as a quick, efficient, cost-effective and useful method in the minds of the individuals seeking counseling, of affected individuals, of physicians and scientists. In this regard the diagnostic panels are a step in the right direction.

Method/Technology

Next-generation sequencing stands for high-throughput sequencing and allows sequencing of up to 100 billion bases within days. A complete human genome can be sequenced with an average coverage of 30 (i.e., each base is read 30 times). Various technology platforms for high-throughput sequencing are commercially available; the main suppliers include Roche, Illumina and Applied Biosystems/Life Technologies. While the Roche platform achieves the comparatively lowest throughput of 400 million bases per run, it offers advantages in regard to the length of the sequenced fragments (approx. 400 bases). Illumina and Applied Biosystems/Life Technologies with up to 100 billion bases achieve far higher throughputs, but with a shorter read length (50 to 100 base pairs). Since all three platforms are based on different sequencing strategies, which we cannot go into here, each of them is also suited for different objectives. The “Sequencing by Ligation” method used by Applied Biosystems/Life Technologies on its SOLiD platform delivers the highest throughput with by far the lowest error rate. This is of great importance for diagnostic applications, since all variants found in high-throughput sequencing are verified conventionally, i.e., via the Sanger method, and the lower the initial error rate the more feasible and safe it will be to introduce high-throughput sequencing into the human genetics practice.

The process of high-throughput sequencing is as follows. A patient’s genomic DNA is obtained from blood or tissue, fragmented randomly by sonication and subsequently equipped with adaptors. The fragments to be sequenced are fished out with complementary RNA baits that are linked to magnetic beads (SureSelect Method by Agilent). The enriched DNA is finally amplified. This is called targeted enrichment. Here as well different technologies are available in the marketplace but cannot be discussed in any detail in this paper. The method used to perform a large number of amplifications in parallel is called EmulsionPCR. This EmulsionPCR is a special kind of PCR that takes place on beads in an aqueous droplets separated by an oil phase. These beads, equipped with several ten thousands of universal PCR-primer molecules, are magnetic and can later on separated by magnetic forces. The enriched DNA is bound via its attached adaptors to the primers on the beads. One DNA fragment is then multiplied by means of PCR in one bead per aqueous droplet at a time. The newly formed fragments bind to additional primers on the same bead. Following breaking the emulsion, those beads, on which a PCR has successfully occurred, are cross-linked by polystyrene beads and separated through centrifugation based on their size and weight of the cross-linked beads when compared to those to which nothing has bound. Then about

700 million beads are deposited on a slide, which in turn is clamped into the high-throughput sequencer.

Sequencing occurs through detection of light signals that are given off by hybridizing octamers (8 nucleotides with one specific fluorescence signals). The octamers contain two nucleotides that code a color space and gives off one of four fluorescent signals immediately after binding, which are photographed by a digital camera with a CCD sensor. The color signals are then separated and a further binding cycle can be initiated until approx. 50 nucleotides can be converted in a series of color signals, whereby one color always corresponds to two nucleotides. The staggered multi-step read of the original sequence ensures that each base is read at least twice and variations can be identified reliably. This results in the special reliability of the SOLiD system by Applied Biosystems/Life Technologies in regard to the interpretation of the data. At the end of the analysis the color signals are converted back into the nucleotide sequence that then enters into the continuing analysis. Five to seven days are required for the sequencing step. The subsequent data analysis consists of the following three individual steps: Step 1: Initially the data generated by the sequencer arrive in the form of pictures consisting of individual color dots, whereby one particular color dot corresponds to two nucleotides. The color dots are examined by the software for their intensity and quality, both necessary for definitely determining the nucleotides. A software supplied by the manufacturer converts the color dots into the color space sequence coded by the color. The automatic comparison with the reference sequence converted into color space, in this case the human genome, takes place in Step 2. The individual 50 color space base pair long fragments are compared to the reference sequence and the color spaces are displayed by side-by-side (alignment). This two base pair encoding of the SOLiD system allows the quick and easy recognition of miscalls and the clear differentiation between miscalls and actual sequence variations. All true deviations from the reference sequence are transmitted and verified via Sanger sequencing. The last and most difficult step involves the interpretation of the data.

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Sexual Development

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New Technologies for the Identification of Novel Genetic Markers of Disorders of Sex Development (DSD)

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Key Words
Comparative genomic hybridization · Disorders of sex development · High-throughput sequencing · Next generation sequencing

Abstract
Although the genetic basis of human sexual determination and differentiation has advanced considerably in recent years, the fact remains that in most subjects with disorders of sex development (DSD) the underlying genetic cause is unknown. Where pathogenic mutations have been identified, the phenotype can be highly variable, even within families, suggesting that other genetic variants are influencing the expression of the phenotype. This situation is likely to change, as more powerful and affordable tools become widely available for detailed genetic analyses. Here, we describe recent advances in comparative genomic hybridisation, sequencing by hybridisation and next generation sequencing, and we describe how these technologies will have an impact on our understanding of the genetic causes of DSD.

The term 'disorders of sex development' (DSD) has been defined as 'congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical' [Hughes et al., 2006]. DSD covers a wide spectrum of phenotypes. 46,XY DSD includes 46,XY complete or partial gonadal dysgenesis, or undervirilisation or undermasculinisation of an XY male due to defects in androgen synthesis or action. 46,XX DSD includes gonadal dysgenesis, or more commonly overvirilisation or masculinisation of an XX female due to androgen excess. Ovotesticular DSD refers to an individual with both ovarian and testicular material present in the same or different gonads, and 46,XX testicular DSD refers to an XX male with testes. Other forms of DSD include cloacal extrophy, severe hypospadias, vaginal atresia, and as part of other conditions such as Mayer-Rokitansky-Kuster-Hauser syndrome, Smith-Lemli-Opitz syndrome or genito-palato-cardiac syndrome [Porter, 2008; Sultan et al., 2009]. Although there have been considerable advances in our understanding of the genetic factors involved in gonadal differentiation in the last 20 years (fig. 1), it has been estimated that a molecular diagnosis is made in only around 20% of DSD, except in cases where the biochemical profile indicates a specific steroidogenic block [Hughes et al., 2006].

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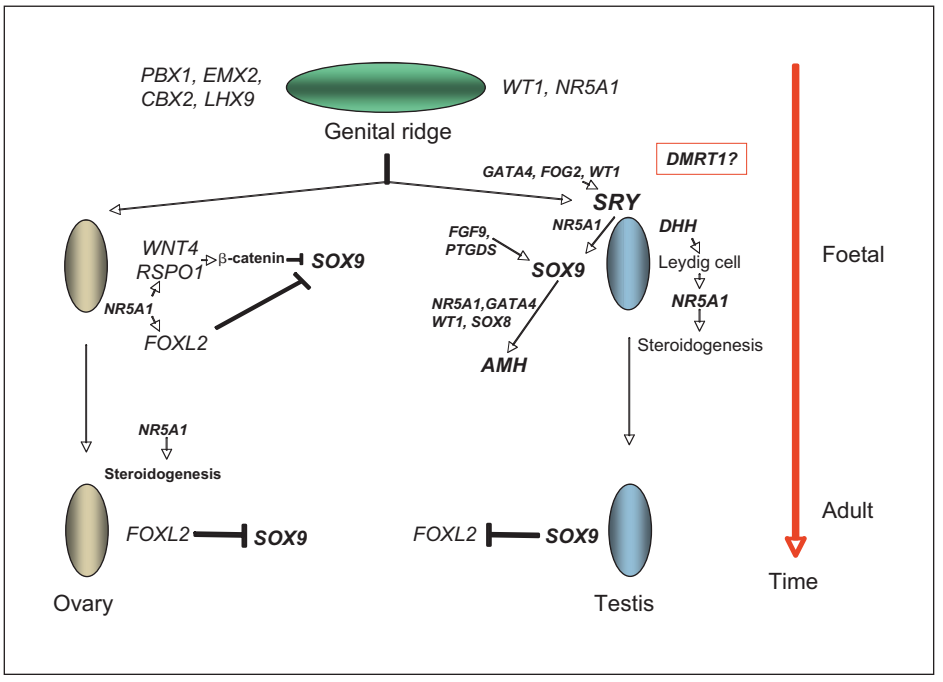

Fig. 1. The molecular and genetic events in mammalian sex determination and differentiation. The bipotential genital ridge is established by several genes including *NR5A1* [Wilhelm et al., 2007; Sekido and Lovell-Badge, 2009]. In the XY gonad the activation of *SRY* expression, possibly initiated by GATA4/FOG2/NR5A1/WT1, leads to the upregulation of *SOX9* expression via a synergy with NR5A1 [Sekido and Lovell-Badge 2008, 2009]. In the XX gonad, the supporting cell precursors accumulate β-catenin in response to RSPO1/WNT4 signalling and repress *SOX9* activity [Schlessinger et al., 2010]. Once SOX9 levels reach a critical threshold, several positive regulatory loops are initiated, including autoregulation of its own expression and formation of feed-forward loops via FGF9 or PGD2 signalling [Sekido and Lovell-Badge, 2009]. At later stages, FOXL2 may repress *Sox9* expression [Uhlenhaut et al., 2009]. In the testis, SOX9 promotes the testis pathway, including Amh activation, and it also probably represses the ovarian genes *Wnt4* and *Foxl2* [Sekido and Lovell-Badge, 2009; Uhlenhaut et al., 2009; Schlessinger et al., 2010]. *DMRT1* controls sex determination in some species of fish and may be the master sex-determining switch in birds, but its role in mammalian sexual development is unclear [Wilhelm et al., 2007; Smith et al., 2009]. Much of this data has been generated from studies in mice.

Identification of Novel Genetic Factors by Comparative Genomic Hybridisation

Comparative genomic hybridisation (CGH) was first described in 1992 as an approach to interrogate cancer genomic DNA using metaphase chromosomes as probes [Kallioniemi et al., 1992]. The strategy of the technique was to differentially label the DNA isolated from a test and reference cell population with different fluorochromes and to cohybridise the labelled samples to a metaphase spread from a reference cell. The relative hybridisation intensity of the test and reference signals at a given location is then proportional to the relative copy number of those sequences in the test and reference genomes. If the reference genome is normal, then increases and decreases in the intensity ratio directly indicate DNA copy number variation in the genome of the test cells. The ratio of the intensities of the 2 fluorochromes reflects the copy number differences between the cells of interest and the control cells.

The major technical challenge of array CGH is the generation of hybridisation signals that are sufficiently intense, specific and quantitative so that copy number changes can be accurately and reliably detected. In the late 1990s, array CGH was first developed by spotting DNA from large-insert clones such as BACs (bacterial artificial chromosomes) and cDNAs onto microarray slides [Solinas-Toldo et al., 1997; Pinkel et al., 1998]. Array elements made from genomic BAC clones (complexity 100–200 kb) typically provide more intense signals than do elements with shorter sequences such as oligonucleotides, so could be analysed by early detection systems. However, the advantage of smaller array elements, such as oligonucleotides is the much higher genomic res-

olution (often ×100) if measurement precision can be maintained. The most recent advancement in CGH microarrays has been the use of oligonucleotide sequences as probes [Lucito et al., 2003] (fig. 2) and the development of genotyping arrays from low-resolution SNP (single nucleotide polymorphism) arrays to high-resolution hybrid arrays that integrate both SNP and CNV (copy number variation) probes [Bignell et al., 2004]. Now many different oligoarray platforms are available for CGH analyses from various companies including Nimblegen (www.nimblegen.com) and Agilent (www.agilent.com) with ever increasing levels of resolution. In addition, several recent Next Generation Sequencing (NGS) approaches also provide information on structural variation (see below).

The recent impact of these technologies on medical genetics has been substantial [Zhang et al., 2009]. These advances include the detection of subtle changes in chromosome architecture associated with cancers [Beroukhim et al., 2010], severe developmental delay [Girirajan et al., 2010], epilepsy [de Kovel et al., 2010], and schizophrenia [Glessner and Hakonarson, 2009]. Like many developmental processes, human sex development is sensitive to gene dosage effects, and duplication and deletion events in the human genome are associated with DSD. For example, deletions of terminal 9p24 are associated with varying degrees of 46,XY gonadal dysgenesis in around 70% of XY individuals with this chromosomal change [Barbaro et al., 2009]. A number of reports have described deletions of terminal 10q associated with 46,XY gonadal dysgenesis together with somatic anomalies. The gene responsible for the gonadal phenotype remains unidentified although *EMX2* has been suggested as a likely candidate [Ogata et al., 2000]. Deletions and duplications of chromosome 22q11.2 have been described in 3 cases of 46,XX *SRY*-negative testicular DSD [Aleck et al., 1999; Erickson et al., 2003; Seeherunvong et al., 2004]. A microduplication of chromosome 17q, including the *SOX9* gene has been reported in a 46,XX man with ambiguous external genitalia and otherwise unremarkable phenotype [Huang et al., 1999]. Deletion of the dosage sensitive gene *NR0B1*, encoding DAX1 on chromosome Xp21.2 results in congenital adrenal hypoplasia (AHC), whereas an *NR0B1* duplication in 46,XY individuals leads to gonadal dysgenesis and a female phenotype [Bardoni et al., 1994]. Duplications of *NR0B1* gene have been described in a small number of other cases of 46,XY gonadal dysgenesis. Duplication of 1p has also been associated with 46,XY gonadal dysgenesis although the gene(s) responsible is not known [Wieacker and Volleth, 2007].

CGH analysis is beginning to reveal novel rearrangements associated with 46,XY and 46,XX DSD. This approach has been used to physically map translocation breakpoints in a case of 46,XX ovotesticular DSD associated with a 12;17 translocation upstream of the *SOX9* gene [Refai et al., 2010]. An inherited 960 kb deletion, positioned 517 kb to 1.477 Mb upstream of the *SOX9* gene, was detected by oligoarray CGH in a XY DSD child with acampomelic dysplasia and in her affected mother [Lecointre et al., 2009]. In both these examples, the rearrangements may have lead to the deregulation of *SOX9* expression. A large 3 Mb deletion of 9q34 that includes the *LMX1B* and *NR5A1* genes was identified using an array CGH approach in a 46,XY girl with clinical features of genitopatellar syndrome and ovotesticular DSD [Schlaubitz et al., 2007]. More recently a smaller 970 kb deletion that included the *NR5A1* gene (encoding steroidogenic factor-1) was also detected in a 46,XY DSD girl without skeletal features [van Silfhout et al., 2009]. CGH analysis has also been used to define deletions on 11p13 and 9p24 associated with syndromic forms of 46,XY DSD [Le Caignec et al., 2007; Vinci et al., 2007].

CGH has successfully identified duplications of *NR0B1* associated with 46,XY complete gonadal dysgenesis in the absence of somatic anomalies [Barbaro et al., 2007] and a unique 257 kb deletion located 250 kb upstream of *NR0B1* in a 21-year-old girl with 46,XY complete gonadal dysgenesis [Smyk et al., 2007]. This deletion presumably results in deregulation of *NR0B1* expression.

Aside from the detection of subtle rearrangements of genetic factors known to be involved in gonadal determination and differentiation, CGH analyses offer the possibility to detect new genes involved in these processes. CGH analyses revealed a novel recurrent 15q24 microdeletion syndrome (1.7–3.9 Mb in size) that is characterised by growth retardation, microcephaly, digital abnormalities, characteristic facial dysmorphia (high anterior hair line, broad medial eyebrows, hypertelorism, downslanted palpebral fissures, broad nasal base, long smooth philtrum and full lower lip), and genital anomalies in the male (micropenis/hypospadias) [Sharp et al., 2007; Andrieux et al., 2009].

As increasing numbers of subjects with DSD are undergoing analysis using CGH platforms, more pathogenic rearrangements will be reported that may reveal unique insights into the mechanism of gonadal somatic cell fate determination and development.

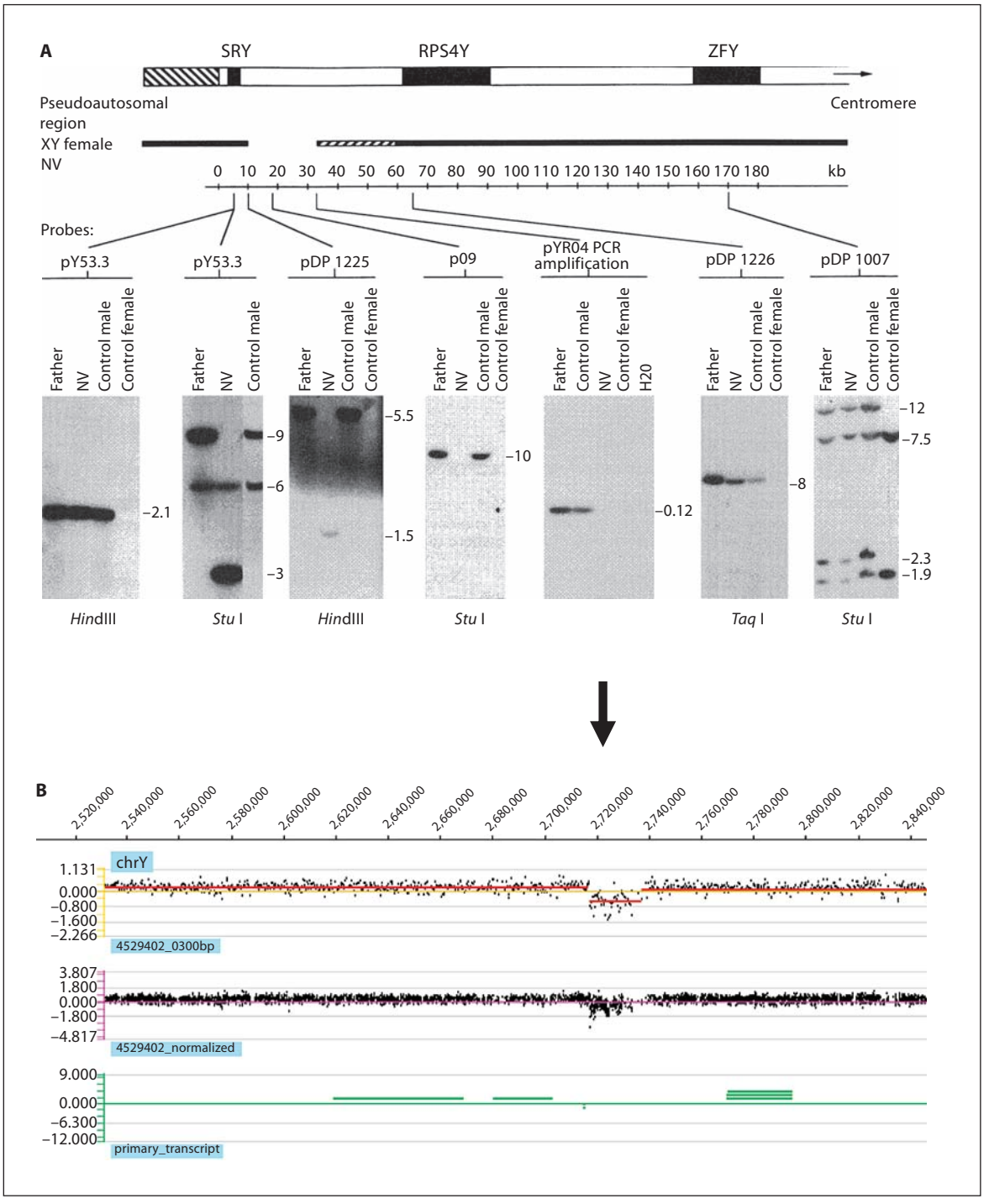

Fig. 2. CGH analysis of a de novo Y chromosome deletion associated with 46,XY gonadal dysgenesis. **A** This case was first published almost 20 years ago and the deletion was detected and the extent of the deletion mapped by Southern blotting [McElreavy et al., 1992b]. **B** The same deletion identified by CGH analysis using a Y chromosome-specific platform (www.nimblegen.com). The genome profile of the patient versus normal male reference genomic DNA is shown for the short arm of the Y chromosome. The x-axis coordinates indicate the relative position of the oligo probes ordered by genomic map position on the Y chromosome. The y-axis shows the log2 ratio shift. The top panel shows window averaging of signals in 300 bp segments. The middle panel shows the normalized signal profile using qspline normalisation. The lower panel indicates the genomic position of transcripts on this region of chromosome Yp.

Identification of Novel Genetic Factors Associated with DSD by Custom Array Sequencing

Diagnostic sequencing of genes known to be responsible for DSD using conventional Sanger sequencing is useful to identify novel mutations associated with gonad development and it is the mainstay of most diagnostic laboratories. However, this approach is both time-consuming and laborious. High-throughput, high-density sequencing using microarray technology offers the possibility of rapidly and accurately sequencing portions of the genome at reduced costs. Several next-generation sequencing technologies are emerging (see below) for whole genome analyses but at present the costs and considerable data handling that are involved means that these approaches will be limited to the research environment for the foreseeable future. In contrast, sequencing by hybridisation is suitable for situations in which a moderate amount of sequence is being analysed (10–300 kb, representing several to tens of genes) in a repetitive manner.

The principle of oligo-hybridisation sequencing is based on the differential hybridisation of target DNA to an array of oligonucleotide probes, thereby decoding its primary DNA sequence. This is most applicable to disorder-specific studies such as DSD where sequences can be compared with an established reference. CustomSeq arrays developed by Affymetrix rely on allele-specific hybridisation for determining the DNA sequence of interest and a single array can be used to sequence up to 300 kb of double-stranded unique DNA sequence. Every base to be sequenced is represented by probes that are 25 bp long and differ only at a single central position [Lipshutz et al., 1999]. The array uses 4 oligomers per base for each of the 2 DNA strands. The 4 oligomers differ at a single central position that could be A, C, G, or T, and they query complementary bases on the DNA strand. The remaining 24 positions are the same for all 4 oligomers and are complementary to the reference DNA sequence being queried.

Sufficient DNA must be generated for hybridisation to the microarray. Current protocols for target-enriched patient material use locus-specific long-range PCR amplification of regions of interest. Long-range PCR minimises the number of reactions required, but genes at multiple loci, with highly dispersed exons, will require many PCR reactions. Long-range PCR products also usually require quantification and normalisation prior to hybridisation (fig. 3). The PCR products are then pooled and fragmented. The DNA fragments are labelled with biotin and subsequently hybridised with the microarray. Finally, the microarray is washed, stained and scanned to measure the fluorescence intensities (fig. 3). Software, such as the GeneChip Sequence Analysis Software (GSEQ; www.affymetrix.com) allows the user to perform sequence analysis of the data to produce the final sequence calls.

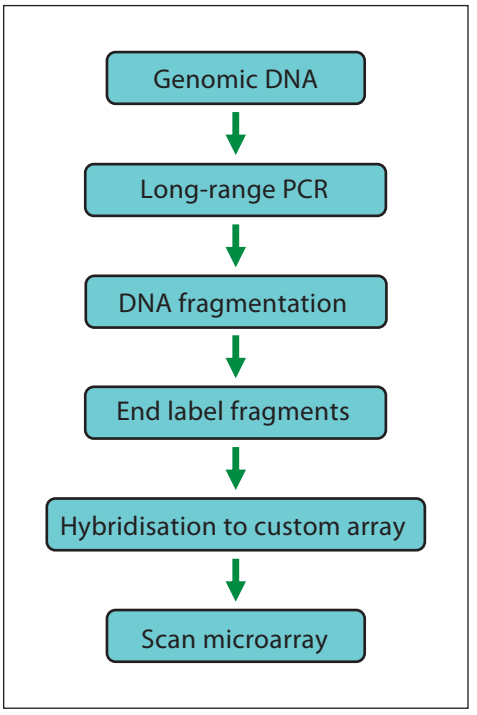

Fig. 3. Schematic overview of procedure involved in sequencing by hybridisation.

Although relatively cheap, sequencing by hybridisation does have the major drawback of false positive calls. These can arise through various mechanisms such as PCR failure or SNP interference. The latter occurs when 2 variants are very close together on the same patient DNA fragment. If there is a homozygous variant, the performance of neighbouring probes, which assume a wild-type base at the variant position, can be poor. Therefore, clusters of SNP calls (i.e. SNPs within 9 bases of each other) should be interpreted with caution. Cross-hybridisation is a cause of false positives, so a single array cannot analyse homologous loci. Repetitive sequences can also generate false positive data, although algorithms have been developed to improve base call reading.

The elimination of false positive calls is essential to reduce the need to perform confirmatory capillary resequencing. Computational algorithms can be developed for specific arrays so that for the detection of rare mutations in clinical disease settings such as DSD, the use of an algorithm that compares a single patient data set to

Table 1. Next generation sequencing approaches

Platform	Sequencing chemistry	Read length (bases)	Gb per run	Run time (fragment library)	System raw accuracy	Consensus base accuracy	Disadvantages
Roche/454 GS FLX titanium	Polymerase	400	0.4–0.6	10 h	99% to 400 bases	99.99% at 15× coverage	Prone to sequence error in homopolymer DNA sequences Cost per run expensive.
Illumina/ Solexa GAII	Polymerase	75–100	20[a]	4 days	98–99%	>99.999% at 20× coverage	Low multiplexing capabilities
Life Technologies, ABI SOLiD 3+	Ligase	50	60[b]	12 days	99.94%	>99.999% at 15× coverage	Duration of run
Helicos Biosciences, Heliscope	Polymerase	32	21	8 days	93–97%	99.995% at 20× coverage	Higher error rates

[a] Paired end sequences. The new Illumina/Solexa HiSeq 2000 generates up to 200 Gb per run.[b] The recent SOLiD™ 4 System generates over 100 gigabases.

data from a group of controls is likely to improve call rates and false positive rates through a statistical comparison of signal strength for each probe. Algorithms may also help to solve another problem confronted by array sequencing technologies, the detection of novel insertion or deletion mutations. However, monoallelic deletions that are larger than a PCR amplicon could be missed due to the normalisation of PCR products prior to hybridisation.

Despite these disadvantages, custom sequencing arrays offer a high-throughput sequencing method that is efficient, fast, automatable, and cost-effective for repetitive resequencing of targeted regions of DNA. Relatively few examples of this approach have been reported in the literature to date. A microarray-based sequencing platform technology to detect sequence alterations in multiple autosomal recessive retinal disease genes was reported with a 99% accuracy and reproducibility [Mandal et al., 2005]. A 16 gene sequencing array has recently been described for studying genetic variation in genes involved in angiogenesis that had a 99.4% sequencing accuracy compared with Sanger capillary sequencing [Szoke et al., 2009]. Similarly, a comparison of 93 worldwide mitochondrial genomes sequenced using either the Affymetrix GeneChip Human Mitochondrial Resequencing Array 2.0 (MitoChip v.2.0) or Sanger sequencing an average call rate of 99.48% and an accuracy of 99.98% for the MitoChip [Hartmann et al., 2009]. The failure to detect some variants was largely due to the inherent difficulty of the array to detect insertions and deletions.

Identification of Novel Genetic Factors by Next Generation Sequencing

The advent of next generation sequencing (NGS) technologies has tremendously reduced the sequencing cost and experimental complexity. NGS technologies also offer improved template coverage, rendering sequencing-based genome analysis more readily available and useful to individual laboratories. These sequencing methods employ massively parallel approaches to sequence several hundred thousand to millions of reads simultaneously [Ansorge, 2009; Metzker, 2010].

The current approaches to NGS falls broadly into 2 types – polymerase based and ligase based [Ansorge, 2009; Metzker, 2010]. The recent use of ligases for massively parallel short-read DNA sequencing of human genomes offers several unique attributes compared to polymerases [Valouev et al., 2008]. In the context of mutation detection, the most important difference is the use of an error-correcting probe-labelling scheme (two-base encoding, or 2BE), which provides error correction concurrent with the color-called alignment of the data (i.e. without having to resequence the reads). There are currently 4 commercially available new-generation sequencing technologies. These are the polymerase based systems of Roche/454, Illumina/Solexa, Helicos HeliScope and the ligase-based Applied Biosystems SOLiD [Margulies et al., 2005; Bentley et al., 2008; Harris et al., 2008; Valouev et al., 2008]. All of these systems produce an abundance of short reads at a much higher throughput than is achiev-

able with the state-of-the-art Sanger sequencer and each have their own unique properties (table 1; see also http://www.wellcome.ac.uk/Education-resources/Teaching-and-education/Animations/DNA/index.htm). Other NGS approaches are on the horizon. These include nanopore sequencing, where individual nucleic acid strands in solution are electrophoretically driven through nanopore-based devices. The narrow pore can ensure that the sequence of nucleic acids can be analysed in their native order without the need for prior amplification or labelling, therefore resulting in increased accuracy and reduced sequencing costs [Branton et al., 2008].

In NGS, the sequencing is only a part of the entire process that includes template preparation, the sequencing reaction itself and the data handling and analyses [Medvedev et al., 2009; McPherson, 2009; Metzker, 2010]. The latter may in fact be more expensive than the costs involved in generating the billions of base pairs of sequence. Considerable challenges remain in terms of data transfer, storage and quality control. Specialised computational analysis and skill are required to align or assemble read data, along with laboratory information management systems for sample tracking and process management [McPherson, 2009].

DNA template for NGS is achieved either by solution-based hybridisation strategies or by solid phase amplification [Vogelstein, 2003; Fedurco et al., 2006]. In emulsion PCR (emPCR), a library of targets is created, and adaptors containing universal priming sites are ligated to the target ends, allowing complex genomes to be amplified with common PCR primers. After ligation, the DNA is separated into single strands and captured onto beads under conditions that favour one DNA molecule per bead. After the successful amplification and enrichment of emPCR beads, millions can be immobilised in a polyacrylamide gel on a standard microscope slide, chemically crosslinked to an aminocoated glass surface, or deposited into individual PicoTiterPlate (PTP) wells (Roche/454) in which the NGS chemistry can be performed [Leamon, 2003; Metzker, 2010].

Solid-phase amplification can also be used to produce randomly distributed, clonally amplified clusters from fragment or mate-pair templates on a glass slide. High-density forward and reverse primers are covalently attached to the slide, and the ratio of the primers to the template on the support defines the surface density of the amplified clusters. Solid-phase amplification can produce 100–200 million spatially separated template clusters (Illumina/Solexa), providing free ends to which a universal sequencing primer can be hybridised to initiate the NGS reaction. The Helicos BioSciences, HeliScope system is also based on polymerase sequencing-by-synthesis of single molecules in which labelled DNA bases are sequentially added to the nucleic acid templates captured on a flow cell (http://www.helicosbio.com). No prior amplification process is involved in this system resulting in a non-bias representation of templates for genome sequencing, although the error rate may be higher than other polymerase-based systems [Harris et al., 2008].

The Roche 454 system uses pyrosequencing technologies to enable the simultaneous sequencing of several hundred thousand DNA fragments, with a read of 400 bp (www.454.com). Pyrosequencing is a bioluminescence method that measures the release of inorganic pyrophosphate into a synthesised DNA strand by converting it into visible light by a series of enzymatic reactions [Ronaghi et al., 1996]. The order and intensity of the generated light is detected and recorded in the form of a peak signal, which reveals the underlying DNA sequence. The Illumina (Solexa) Genome Analyzer (GA) uses sequencing-by-synthesis to generate ~200 million 75–100-bp reads (http://www.illumina.com/systems/genome_analyzer.ilmn). This is a cyclic method that comprises nucleotide incorporation, fluorescence imaging and cleavage. In the first step, a DNA polymerase, bound to the primed template, adds or incorporates just one fluorescently modified nucleotide, which represents the complement of the template base. The termination of DNA synthesis after the addition of a single nucleotide is an important feature of this approach. Following incorporation, the remaining unincorporated nucleotides are washed away. Imaging is then performed to determine the identity of the incorporated nucleotide. This is followed by a cleavage step, which removes the terminating/inhibiting group and the fluorescent dye. Additional washing is performed before starting the next incorporation.

A novel massively parallel sequencing technology based on ligation of oligonucleotides as opposed to sequencing by synthesis has been developed by Applied Biosystems (http://solid.appliedbiosystems.com). In this approach, sequencing is carried out via sequential rounds of probe annealing and ligation with high fidelity and high read quality (fig. 4). Sequence errors are considerably reduced compared to polymerase-based systems because the ligation method is based on probe recognition rather than sequential nucleotide addition. In this system, there are 16 dinucleotide combinations with 4 fluorescent dyes, each dye corresponding to a probe pool of 4

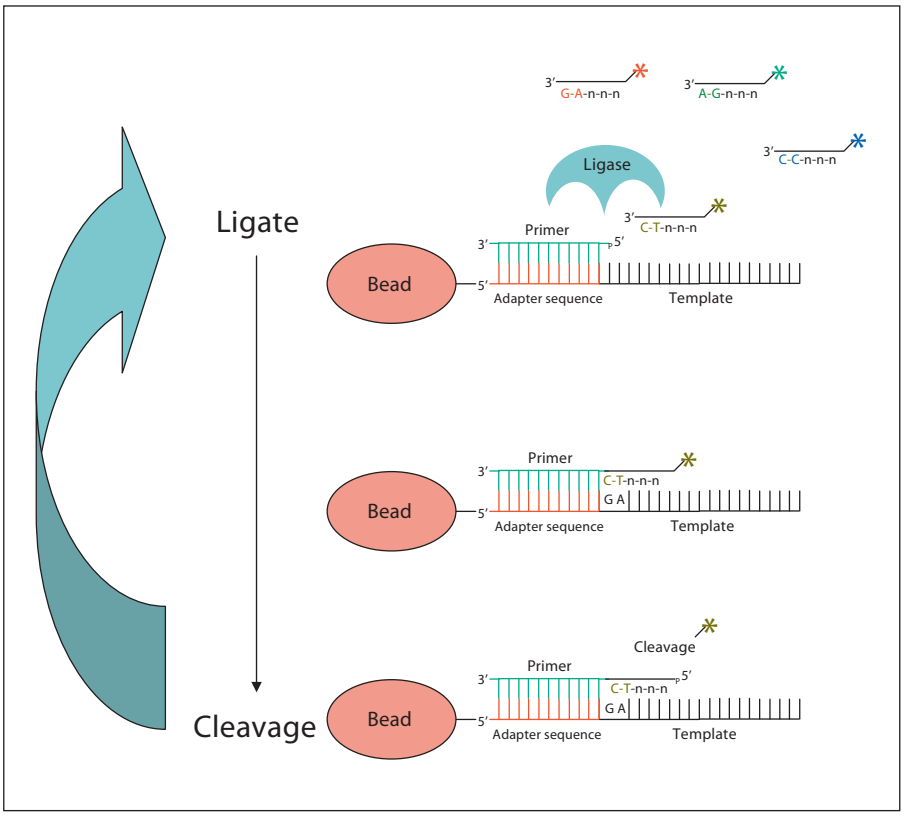

Fig. 4. Schematic representation of SOLiD™ sequencing by ligation. Primers hybridise to the P1 adapter within the library template. A set of 4 fluorescence-labelled di-base probes competes for ligation to the sequencing primer. These probes have partly degenerated DNA sequence (indicated by n) and for simplicity only one probe is shown (labelling is denoted by asterisk). The specificity of the di-base probe is achieved by interrogating every 1st and 2nd base in each ligation reaction. Multiple cycles of ligation, detection and cleavage are performed with the number of cycles determining the eventual read length (modified from http://www3.appliedbiosystems.com).

dinucleotides per pool (fig. 4). Using this dinucleotide, 4-dye encoding scheme in conjunction with a sequencing assay that samples every base, each base is effectively probed by 2 different and independent reactions. The double interrogation of each base causes a true SNP polymorphism to result in a 2 consecutive colour change while a measurement error results in a single colour change. The SOLiD™ (Sequencing by Oligo Ligation and Detection) platform is capable now of producing 400 million 50-bp reads (combined total in 2 independent flow cells). This approach essentially eliminates the possibility of spurious insertions or deletions.

Recently, the SOLiD system has been used to assay nucleotide variation in HapMap samples, in the framework of the 1000 Genomes Project [McKernan et al., 2009]. The 1000 Genomes Project is a collaboration among research groups in the US, UK, China, and Germany to produce an extensive catalogue of human genetic variation that will support future medical research studies (www.1000genomes.org). It will extend the data from the International HapMap Project and will allow genome-wide association studies to focus on almost all variants that exist in regions found to be associated with disease. The genomes of over 1,000 unidentified individuals from around the world will be sequenced using next generation sequencing technologies. Three HapMap samples were sequenced via the ligation-based approach utilised in the SOLiD sequencing system [McKernan et al., 2009]. In one sample with a sequence coverage estimated at 29×, 97% of heterozygotes SNPs and 99.96% of homozygotes SNPs were detected and false discovery rate was negligible [McKernan et al., 2009].

This approach is particularly useful for variation discovery in genetic association studies, where low sequence coverage (5×) and therefore lower cost, may detect a significant portion of the total sequence variation. To detect pathogenic mutations associated with disease a higher coverage is needed. What is the required accuracy for mutation discovery? A single base pair change may result in a DSD phenotype and any error in sequencing can give rise to false positives or false negatives, leading to time-consuming and challenging downstream studies. The raw sequencing accuracy is the accuracy of a single-pass sequencing read. This is an excellent measurement of each method's chemistry, fluorescence readout, process, and base-calling software

(table 1). Ultimately, the more robust the raw reads, the fewer redundant reads are required, keeping the overall costs down. At the time of writing, the SOLiD system shows a high degree of sequence accuracy, even at low coverage, and this may be the system of choice for mutation detection.

Overall substantial cost reductions are associated with NGS technologies when compared with the Sanger method, but sequencing the whole human genome remains expensive for most laboratories. In the search for novel genetic markers of DSD, the medium term may see a more targeted approach. Specific regions of the genome may be enriched for sequencing: for example, sequencing all of the exons in the genome; sequencing specific gene families of interest; or focusing just on large chromosome regions or entire chromosomes that are implicated in disease. Exome capture approaches are available for targeting approximately 33 Mb or ~180,000 coding exons across the human genome. Many platforms also offer capture of miRNAs and non-coding RNA sequences. Selective genomic enrichment of the human exome offers an attractive option for devising new experimental designs aimed at quick identification of potential disease-associated genetic variants. Human exome capture methods are currently based on either custom-designed oligonucleotide microarrays or solution-based hybridisation strategies. Roche/Nimblegen offers microarray capture platforms for solid phase hybridisation for the enrichment of exons [Albert et al., 2007]. The reported capture efficiency for these platforms ranges from 50–90% [Hodges et al., 2007]. Solution-based hybridisation methods for exon capture, such as molecular inversion probes (mIPs) [Porreca et al., 2007; Turner et al., 2009] and biotinylated RNA capture sequences [Gnirke et al., 2009; SureSelect Human All Exon Kit; Agilent Technologies] have also been developed that have capture efficiencies of 70–90%. Raindance technologies (www.raindancetechnologies.com) offers targeted sequence enrichment using a microfluidic device to create aqueous picolitre-volume droplets of forward- and reverse-targeting primers in an oil solution [Tewhey et al., 2009]. In a microfluidic chip, the primer pair droplets and template droplets, which contain fragmented genomic DNA and PCR reagents, are paired together in a 1:1 ratio. An electric field induces the 2 droplets to merge into a single PCR droplet. Around 1.5 million PCR droplets can be collected in a single PCR tube. This approach has been reported to have an 84% capture efficiency with 90% of the targeted bases showing uniform coverage when sequenced with either the Roche/454 or Illumina/Solexa platform [Tewhey et al., 2009]. From a medical perspective, this approach is interesting since it is particularly suitable for working with cells of limited availability, such as stem cells or primary cells from patients [Brouzes et al., 2009].

NGS with exome capture has recently been applied to identify mutations associated with mendelian disorders [Ng et al., 2009, 2010]. Using Freeman-Sheldon syndrome (FSS) as a proof-of-concept, Ng and colleagues performed exome enrichment and sequencing using the Illumina system to identify the causal mutation for FSS. The same group has recently identified mutations in the *DHODH* gene in 4 individuals from 3 pedigrees with Miller syndrome (postaxial acrofacial dysostosis) using exome sequence capture and sequencing to a coverage of 40× with sufficient depth to call variants at approximately 97% of each targeted exome.

In addition, the recent NGS applications not only have the ability to generate huge amounts of sequence data but they also reveal structural variation, thereby bypassing the need for CGH analyses [McKernan et al., 2009].

These recent studies show the power of NGS approaches to detect pathogenic mutations causing disease. This approach also offers the opportunity to identify genetic modifiers. Familial cases of both 46,XY and 46,XX DSD often show considerable variation in the expression of the phenotype, including families where the underlying genetic mutation has been identified [Lourenco et al., 2009; Temel et al., 2007]. This phenotypic variability may be explained by variations in other genes that may interact with or influence the activity of the target gene. This has been shown in a pedigree with hypogonadotropic hypogonadism, where a compound heterozygous *GNRHR* and a heterozygous *FGFR1* mutation were identified [Pitteloud et al., 2007].

When considering these NGS approaches that are currently costly and require considerable bioinformatics analyses, an important question to pose is what would be the cost benefits to the DSD field? The usefulness of this non-a priori approach to identify genetic modifiers has been mentioned above, but are there new genetic factors that when mutated could cause DSD? The reply to this question is yes. For example, almost all cases of non-syndromic ovotesticular DSD remain unexplained although there have been considerable genetic analyses of candidate genes [McElreavey et al., 1992a; Temel et al., 2007]. Despite advances in understanding the mechanisms of mammalian gonad formation, the underlying genetic cause of most cases of 46,XY gonadal dysgenesis remains

unknown [McElreavey and Fellous, 1999]. Mutations in the androgen receptor (AR) gene are the most common genetic cause of 46,XY DSD. However, even in 46,XY underandrogenised subjects with testes who are suspected to have an AR defect, a pathogenic mutation is found only in less than half of the cases [Audi et al., 2010]. In cases of simple hypospadias or cryptorchidism a genetic cause is rarely detected despite epidemiological evidence suggesting a major genetic contribution to these phenotypes [Fukami et al., 2006; Schnack et al., 2008; Köhler et al., 2009]. Recently, a mutation in *Map3k4* was associated with XY gonadal sex reversal in the mouse, suggesting that mutations in mitogen-activated protein kinase (MAPK) signalling pathway may be associated with DSD [McElreavey et al., unpublished data; Bogani et al., 2009].

It is clear that NGS will have a tremendous impact on medicine [Voelkerding et al., 2009]. Exciting developments are rapidly taking place in the field and these are leading to a deeper understanding of variation across individual genomes, genetic variation in tissues within an individual and the identification of disease causing mutations. Sequencing technology is continuing to evolve at an unprecedented pace. The generation of massive data sets of qualitative and quantitative information of both RNA and DNA sequences in a patient sample at a relatively limited cost will transform the field of reproductive disorders by offering novel insights into the genetics and physiology of DSD.

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Erratum

In the article by Bashamboo et al. 'New Technologies for the Identification of Novel Genetic Markers of Disorders of Sex Development (DSD)' (Sex Dev DOI: 10.1159/000314917) an error occurred with one of the author names.
Now it reads 'J. Achermann' but the correct name is 'J.C. Achermann'.

Loss-of-function mutation in *GATA4* causes anomalies of human testicular development

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Approximately 1 of every 250 newborns has some abnormality of genital and/or gonadal development. However, a specific molecular cause is identified in only 20% of these cases of disorder of sex development (DSD). We identified a family of French origin presenting with 46,XY DSD and congenital heart disease. Sequencing of the ORF of *GATA4* identified a heterozygous missense mutation (p.Gly221Arg) in the conserved N-terminal zinc finger of GATA4. This mutation was not observed in 450 ancestry-matched control individuals. The mutation compromised the ability of the protein to bind to and transactivate the anti-Müllerian hormone (*AMH*) promoter. The mutation does not interfere with the direct protein–protein interaction, but it disrupts synergistic activation of the *AMH* promoter by GATA4 and NR5A1. The p.Gly221Arg mutant protein also failed to bind to a known protein partner FOG2 that is essential for gonad formation. Our data demonstrate the key role of GATA4 in human testicular development.

Human disorders of sex development (DSD) are congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical (1). 46,XY DSD includes errors of testis determination and differentiation (complete or partial gonadal dysgenesis), or undervirilization or undermasculinization of an XY male (1). Despite the considerable advances in our understanding of the genetic components of gonad development, the mechanisms involved in human sex determination remain poorly understood. This is reflected in the relative paucity of pathogenic mutations that have been identified in DSD patients. It has been estimated that a molecular diagnosis is made in only 20% of DSD cases, except where the biochemical profile indicates a specific steroidogenic block (1).

GATA4 belongs to the evolutionarily conserved GATA family of six tissue- and organ-specific vertebrate transcriptional regulators, consisting of two zinc fingers (2, 3). The C-terminal zinc finger region is required for the recognition and binding of DNA, and the N-terminal zinc finger region contributes to the stability of this binding (3). The zinc fingers are also crucial for protein–protein interactions with other transcription cofactors (2, 3). In the mouse and human, *GATA4* is strongly expressed in the somatic cell population of the developing gonad before and during the time of sex determination (4). GATA4 cooperatively interacts with several proteins, including NR5A1 and FOG2, to regulate the expression of the sex-determining genes *SRY* (encoding sex-determining region Y), *SOX9* (encoding SRY box 9), *AMH* (encoding anti-Müllerian hormone), as well as key steroidogenic factors, including *STAR* (encoding steroidogenic acute regulatory protein), *CYP19A1* (encoding aromatase), *INHA* (encoding inhibin α-subunit), and *HSD3B2* (encoding hydroxy-δ-5-steroid dehydrogenase, 3 β- and steroid δ-isomerase 2) (5–7).

Mice lacking *Gata4* die in utero due to profound abnormalities in ventral morphogenesis and heart tube formation (8, 9). In the human, mutations in *GATA4* are associated with congenital heart defects (CHD), including atrial septal defects, ventricular septal defects, pulmonary valve thickening, or insufficiency of the cardiac valves (10–13). In all of the cases of CHD associated with mutations in *GATA4*, other organs were reported as normal.

The critical role for GATA4 in gonadal development is highlighted by *Gata4*^{ki} mice that have a p.Val217Gly mutation in the N-terminal zinc finger domain (14). This knock-in mutation abrogates the interaction of GATA4 with the cofactor FOG2, and these animals display severe anomalies of testis development (15, 16). FOG2 may act as a transcriptional repressor or activator, depending on the cellular and promoter context. Mice lacking *Fog2* exhibit a block in gonadogenesis, and a translocation involving *FOG2* in the human is reported to be associated with male hypergonadotropic hypogonadism (15–17). In vitro FOG2 represses GATA4-dependent transcription of *AMH* in primary Sertoli cell cultures (18). Although the mechanism of FOG-2 and GATA4 interaction in the gonad is not well defined, it is essential that a direct physical interaction between GATA4 and FOG2 be maintained, because abrogation of the same results in abnormal testis development in mice (14–16).

NR5A1, also termed Ad4 binding protein (Ad4BP) or steroidogenic factor 1 (SF-1), is a key transcriptional regulator of genes involved in sexual development, many of which are also regulated by GATA4 (19–21). Mutations in *NR5A1* are associated with 46,XX ovarian insufficiency and 46,XY DSD (22, 23). GATA4 functionally interacts with NR5A1 in primary Sertoli cell cultures to positively regulate the expression of *AMH,* through two complementary mechanisms, either by binding to its site on the *AMH* promoter or by direct interaction with NR5A1 when either NR5A1 alone or both GATA4 and NR5A1 are bound to their respective sites on the *AMH* promoter (18). Mutations in *NR5A1* may cause 46,XY DSD through a lack of appropriate interaction with GATA4 (24). No mutations in *GATA4* have been reported in association with human cases of DSD. The absence of an associated gonadal anomaly in the reported cases of CHD associated with *GATA4* mutations may also be due to the ability of the mutated GATA4 proteins to retain the ability to interact with either FOG2 or NR5A1 or both (15–18, 25).

Here, we describe a familial case of 46,XY DSD and CHD associated with a heterozygous GATA4 p.Gly221Arg mutation. This mutation in *GATA4* is associated with 46,XY DSD, and the data suggest that DNA-binding activity of GATA4 is essential for transcriptional activation of the *AMH* promoter.

Results

Clinical Spectrum in a French Family with 46,XY DSD and CHD. The family studied is of French ethnic origin (Fig. 1*A*). The three affected male patients had 46, XY karyotype and normal parameters at birth. The index case (IV.2) was referred at birth for ambiguous external genitalia (Table 1). Genitography showed a 15-mm-diameter cavity communicating with urethra at the level of veru montanum and no uterus. Plasma concentrations of adrenal steroids before and 1 h after synthetic adreno-

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The authors declare no conflict of interest.

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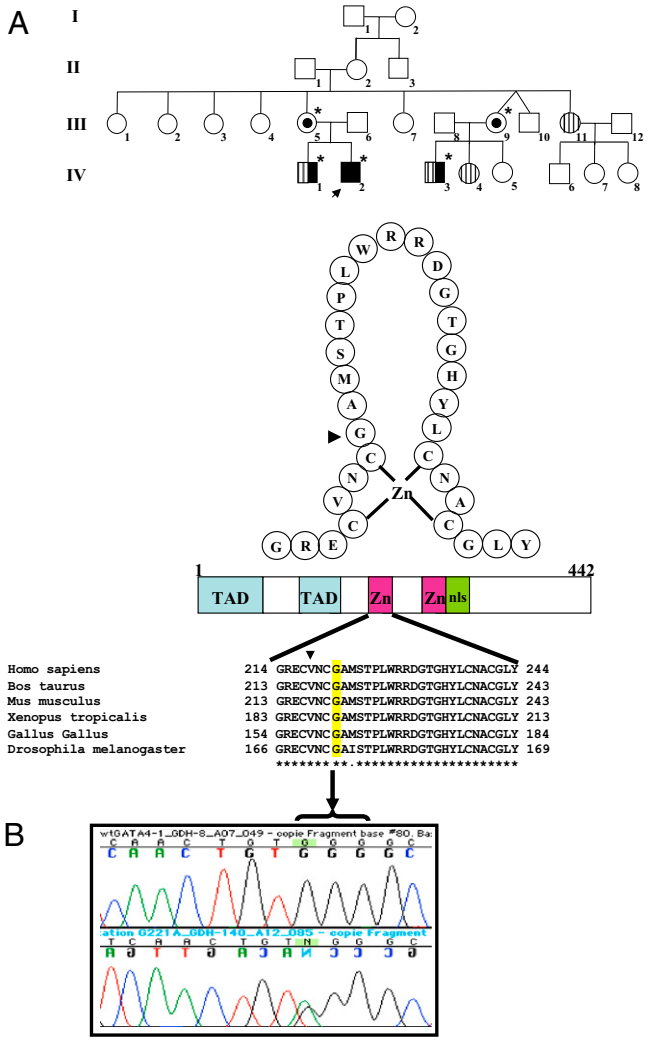

Fig. 1. (*A*) Pedigree of familial case with 46,XY DSD. Squares represent male family members, and circles represent female family members. Solid squares represent affected 46,XY DSD subjects who were raised as boys. Striped circles and partially striped squares indicate individuals with cardiac anomalies. Symbols containing a black dot represent apparently unaffected carriers of the mutation. The index patient is indicated with an arrow. The asterisk indicates individuals who were screened for mutations in the *GATA4* gene. Individuals I.2, II.2, and II.3 had implantation of a cardiac pacemaker. Case IV.2 developed hemolytic and uremic syndrome at 7 y, which resolved spontaneously. Cases II.2, III.2, and IV.1 had benign hypervascularized thyroid nodules, all with normal thyroid function. (*B*) The localization of the GATA4 p.Gly221Arg mutation in relation to the protein. (*Top*) Schematic representation of the functional domains of the GATA4 protein is shown with an arrow indicating the position of the p.Gly221Arg mutation. The N-terminus transcription activation domains (TAD) and the DNA-binding domain containing two zinc-finger (ZN) motifs are indicated. The nuclear localization signal (NLS) lies distal to the second zinc finger. (*Middle*) Sequence alignment of the proximal zinc finger of human GATA4 protein with other species shows a high degree of amino acid conservation. The position of the mutation is highlighted in yellow, and the arrow indicates the position of the p.Val217Gly *Gata4*^{ki} mutation in the mouse Gata4 protein that abolishes interaction with the Fog2 protein and is associated with gonadal anomalies. (*Bottom*) A representative chromatogram is shown of the heterozygote mutation in DNA from the proband.

corticotropic hormone were within the normal range. At 40 d and at 1 y, the patient was given 30 mg of testosterone enanthate i.m. four times each 14 d, leading to an increase in phallus size and to pubic hair development. At 1 y, he was operated on for cryptorchidism. This revealed a bilateral disjunction between the epididymus and testis and a perforation in the left albuginea. At 1.5 y, urethroplasty was performed. At 10 y, testicular ultrasonography revealed diffuse calcifications. At 20 y, he is a sexually active male with height of 186 cm and weight 77 kg. He has azoospermia. There was no clinical evidence of heart anomalies by echocardiogram assessment (data available upon request).

His brother (IV.1) had decreased phallus length and inguinal hernias at birth (Table 1). He was operated on at 2.8 y for bilateral inguinal hernias, and a minor systolic murmur was noted, suggesting an atrial septal defect. At 10.5 y, he was evaluated because of the ambiguous external genitalia seen in his brother (IV.2). His external genitalia were normal with pubic hair development. At 12.5 y, testicular ultrasonography revealed calcifications. At 18 y, a systematic cardiac evaluation was performed because of the previous systolic murmur. Doppler echocardiography revealed a normal left ventricle and a slightly dilated hypokinetic right ventricle. There were no cardiac rhythm anomalies, pulmonary pressure was normal, and there was no evidence of atrial septal defect. At 22.8 y, he is a sexually active male with height of 178 cm and weight 68 kg.

Case IV.3 was referred at 26 d for ambiguous external genitalia (Table 1). Genitography showed a cavity of 15 × 8 mm communicating with the urethra at the level of veru montanum and no uterus. A minor systolic murmur was noted that did not require medical intervention. At 7 mo, he was operated on for bilateral inguinal hernias. The gonads measured 4 mm at their largest and were surrounded by an epididymus and vas deferens. They were removed, and histological examination revealed bilateral dysgenetic testes. At 9 mo, he was given testosterone, and urethroplasty was performed at 2 y. At 16 y, his height is 177 cm and weight 60 kg and he is undergoing substitutive testosterone replacementtherapy.

Case IV.4 was diagnosed at birth with tetralogy of Fallot and received corrective surgery. She is now 10 y and in good health. Case III.11 was noted at birth to have congenital cyanotic heart disease. This was well tolerated, and medical intervention was not required. She has two healthy daughters and one son with no history of medical intervention.

Heterozygous *GATA4* Mutation in the N-Terminal Zinc Finger. We identified a heterozygous c.661G<A transition that is predicted to result in a p.Gly221Arg mutation in the highly conserved N-terminal zinc finger domain of GATA4 (Fig. 1*B*). This mutation was initially detected in the proband (IV.2) and was identified in affected brother (IV.1) and his affected cousin (IV.3). Further investigation of the family revealed that the apparently unaffected mothers (III.5 and III.9) also carried the mutation. DNA samples from other family members were not available for study. Sequencing of the entire *GATA4* ORF in 450 unrelated, healthy control samples of European descent that included 342 individuals of French ancestry showed no rare allelic variants, including the mutation in this family. To exclude a role of other known sex-determining genes, we sequenced the coding regions and intron-exon boundaries of the Doublesex and Mab-3-related transcription factor 1 (*DMRT1*), sex-determining region Y (*SRY*), SRY-box 9 (*SOX9*), nuclear receptor subfamily 5, group A, member 1 (*NR5A1*), nuclear receptor subfamily 5, group A, member 2 (*NR5A2*), Wilms tumor 1 (*WT1*), and mastermind-like domain containing 1 (*MAMLD1*) in case IV.2 and his affected brother case IV.1. Pathogenic mutations were not detected in any of these known sex-determining genes. Discrete chromosomal rearrangements are known to be associated with 46,XY DSD (26). High-resolution comparative genomic hybridization indicated that case IV.2 did not carry any rearrangement in his genome known to be associated with 46,XY DSD (such as del9p, del10q, dupXp, etc.).

GATA4 p.Gly221Arg Variant Does Not Affect Nuclear Localization but Exhibits Altered DNA Binding and Transactivation Abilities. To assess the impact of the GATA4 p.Gly221Arg mutation on cellular localization, a protein expression vector was constructed by cloning mouse full-length *GATA4* cDNA into the pIRES-hrGFP II vector. The *GATA4* expression vector containing the p.Gly221Arg

Table 1. Phenotypes and gonadal function in the three 46,XY DSD patients with a mutation in the *GATA4* gene

					Hormonal data									
						ACTH stimulation test		Testosterone, ng/mL		DHT ng/mL		FSH, U/L		Luteinizing hormone, U/L	
Subject no.	Initial clinical presentation	Age at evaluation	Testis vol, mL	Phallus size, mm	AMH ng/mL	Basal	After ACTH	Basal	After hCG	Basal	After hCG	Basal	Peak	Basal	Peak
IV.2	Ambiguous genitalia: fused hypoplastic labioscrotal fold, perineal hypospadias, hypoplasia of corpus cavernosum, bilateral inguinal hernia containing gonads	7 d	ND	13	ND	0.06	0.05	0.0	0.25	0.06	0.05	3.9	7.6	0.2	0.9
		40 d	ND	ND	ND	Androstenedione, 1.9 ng/mL DHEAS, 790 ng/mL 17-OH-pregnenolone, 3.9 ng/mL	2.3 ng/mL 1,050 ng/mL 29 ng/mL	ND	ND	ND	ND	ND	ND	ND	ND
		3.5 m	ND	25 × 10	3.2	ND	ND	0.7	1.9*	ND	ND	ND	ND	ND	ND
		2 y	1	43 × 15	6.2	ND	ND	ND	ND	ND	ND	6.9	26	0.7	8
		10 y	2	ND	6.2	ND	ND	<0.37	ND	ND	ND	12	ND	0.34	ND
		14 y[†]	6	ND	ND	ND	ND	3	ND	ND	ND	41	ND	16	ND
		20 y	7	75 × 25	0.3	ND	ND	4	ND	ND	ND	27.5	ND	10.5	ND
IV.1	Microphallus; bilateral inguinal hernia containing gonads	10.5 y	7	50 × 10	ND	ND	ND	0.32	ND	0.17	ND	8.6	ND	0.7	ND
		12.5 y[‡]	8.4	ND	5.4	ND	ND	3.4	ND	ND	ND	23	ND	4.1	ND
IV.3	Ambiguous genitalia: fused labioscrotal folds, hypospadias, bilateral inguinal hernia containing gonads[§]	26 d	0.4	8 × 5	ND	Androstenedione, 0.6 ng/mL; DHEAS, 250 ng/mL; 17-OH-pregnenolone, 1 ng/mL	ND	0.2	0.3 0.5*	0.05	0.08	17.5	58	3.5	22

*Combined hCG and hMG stimulation.
[†]Spontaneous puberty began at 12 y and was complete by 14 y.
[‡]Pubertal development began at 12.5 y.
[§]Histology of gonads removed at 7 mo showed abundant conjunctive tissue together with tubule-like structures containing Sertoli cells and rare spermatogonia. Leydig cells were not visible. ND, not determined. ACTH test: basal blood sample was taken at 8 AM immediately followed by i.m. infusion of tetracosactide acetate (Synacthen; 0.125 mg before 18 mo or 0.25 mg after 18 mo); second sample was taken 60 min after the infusion. The normal ranges for hormonal values in boys are FSH U/L: basal 1–3 y, 0.4–1.5 peak 2.3–6.9; 4–9 y, <0.3–2.4; 10–13 y. P1: <0.3–3; P2: 11–15 y, <0.3–3.5; P3: 13–18 y, 0.6–4.8; P5: 0.8–4.4, adult 3–7. LH U/L: basal 1–3 y, <0.3–1.3; peak 1.4–6; 4–9 y, 0.2–1.9; 10–13 y. P1: 0.2–2.1; P2 11–15 y 0.2–1.9; P3 13–18 y 0.2–2.2; P5 0.5–5; adult 3–8. Total testosterone (ng/mL): 0–4 mo: 0.1–3.5; 4 mo to 11 y: <0.5; 13 y: 0.2–3; adult: 3.5–7.5. AMH ng/mL. <15 d: 32.1 ± 8.3; 15 d to 1 y: 65.1 ± 13; 1–4 y: 69.9 ± 9.2; 4–7 y: 61.3 ± 8.4; 7–9 y: 47 ± 6.6; P1: 34.9 ± 3.7; P2: 22.2 ± 3.5; P3: 11 ± 3.9; P4–5: 6.7 ± 1.9. Androstenedione, 0.46 ng/mL (range 0.1–1.0); DHEAS, 400 ng/mL (range 70–1,300); 17-OH-pregnenolone, 3 ng/mL (range 0.62–8.3); after ACTH stimulation peak, 16 ng/mL (range 9–32).

variant was generated by site-directed mutagenesis of the WT *GATA4* construct. The presence of the IRES element in the vector allows the coexpression of either WT GATA4 or GATA4 p.Gly221Arg with hrGFP (human recombinant GFP) from a single transcriptional unit. Both the WT and mutant GATA4 proteins (green), when expressed in human embryonic kidney HEK 293-T cells, showed strong nuclear localization (Fig. 2*A*).

The p.Gly221Arg mutation falls within the highly conserved N-terminal zinc finger domain, a region that is known to contribute to the stability of DNA binding. We hypothesized that as a consequence of this mutation, the protein may have altered DNA binding activity. To test this hypothesis, we performed EMSA, which showed WT GATA4 binding to the GATA4-responsive element on the *AMH* promoter, whereas the p.Gly221Arg mutant failed to bind to this motif (Fig. 2*B*). The mutant GATA4 protein had altered biological activity in luciferase reporter assays driven by GATA4-dependent gonadal promoters. A quantitative reduction in transactivation of the *AMH* promoter was observed in reporter assays using HEK293-T cells (Fig. 2*C*). These in vitro functional assays demonstrated that p.Gly221Arg mutation may lead to a functional disruption of the GATA4 protein and may affect the regulation of its downstream target genes during testis development and function.

GATA4 p.Gly221Arg Physically Interacts with NR5A1 but Fails to Interact with FOG2. To further investigate the functional consequences of the p.Gly221Arg mutation on GATA4 protein, we analyzed the ability of the WT and mutant GATA4 to physically interact with the protein partners NR5A1 and FOG2. The GATA4 WT, GATA4p.Gly221Arg, NR5A1, and FOG2 proteins were in vitro translated. Equivalent amounts of WT and mutant GATA4 proteins were fractionated on a 10% SDS/PAGE gel and transferred to nitrocellulose membrane. The immobilized proteins were incubated with in vitro translated bait protein (either NR5A1 or FOG2), and bound bait was detected with antibait antibodies, followed by secondary antibody conjugated with HRP and visualized by chemiluminescence. In multiple experiments, the far Western analyses revealed that both the WT and mutant GATA4 proteins retained their ability to interact with NR5A1 (Fig. 3*A*), but the mutant p.Gly221Arg GATA4 protein failed to interact with the FOG2 protein (Fig. 3*B*). This is a similar finding to the *Gata4*^{ki} mice that have a p.Val217Gly mutation in the N-terminal zinc finger domain that also lacks the ability to interact with FOG2 (14, 15).

GATA4 p.Gly221Arg Does Not Synergize with NR5A1 to Stimulate Reporter Gene Activity. GATA4 binds to NR5A1 to synergistically enhance the expression of *AMH* by regulating its promoter (24). We studied the ability of p.Gly221Arg mutant to retain this synergy. Although the mutant protein retained the ability to physically interact with the NR5A1 protein, it showed a statistically significant reduction ($P = 0.00652300$) in the ability to boost *AMH* reporter gene activity above the levels achieved by NR5A1 alone in HEK 293T cells (Fig. 3 *A* and *C*) or using the murine ES cell line E14 (data available upon request). The mutation therefore disrupts the synergy between GATA4 and NR5A1 for activation of the *AMH* gene.

GATA4 p.Gly221Arg Does Not Show Dominant Negative Activity. The mutation in this family is heterozygous, and we investigated if the mutant GATA4 p.Gly221Arg may be pathogenic by being dominant negative on the WT GATA4 protein function. The GATA4 p.Gly221Arg mutant does not inhibit or interfere with the ability of WT GATA4 allele to transactivate the *AMH* promoter (Fig. 4*A*) or suppress the synergy between WT GATA4 and NR5A1 to transactivate the *AMH* promoter (Fig. 4*B*), indicating that the mutant protein does not exhibit dominant negative activity.

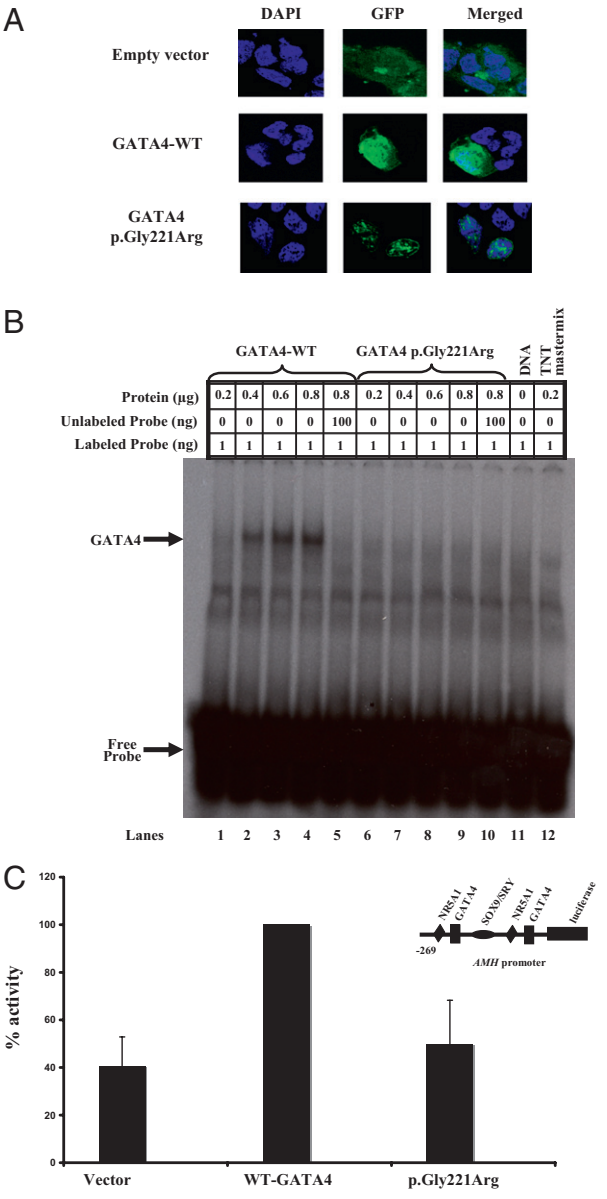

Fig. 2. (*A*) Nuclear localization of GATA4. Human embryonic kidney HEK 293-T cells were transfected with WT or mutant GATA4 GFP-labeled expression vectors. The cellular localization of both WT and mutant GFP–GATA4 fusion proteins (green) showed strong nuclear localization. (*B*) DNA binding of *GATA4*. EMSA on increasing concentrations of in vitro-translated WT (lanes 1–5) or mutant GATA4 (lanes 6–10) was performed using ^{32}P-radiolabeled probe corresponding to the GATA4 response elements in the *AMH* promoter. WT GATA4 binds to the *AMH* promoter, and the binding is specific as indicated by the effective competition of a 100-fold excess of the unlabeled AP-1 probe (lane 5). The mutant GATA4 p.Gly221Arg protein does not bind to the *AMH* promoter. Controls consist of the probe alone (lane 11) and master mix from the TNT™ coupled transcription-translation system (lane 12; Promega). (*C*) Transcriptional activation of *AMH* promoter construct by *GATA4*. The transcriptional activities of WT GATA4 and mutant GATA4 p.Gly221Arg were studied using the human *AMH* promoter as a reporter following transfection in HEK293-T cells. The data shown here represent the mean ± SEM of three independent experiments, each of which was performed in triplicate. The human *AMH* reporter construct was transfected into HEK293-T cells with either the WT GATA4 or the mutant GATA4 expression vector. The results are expressed as a percentage of WT GATA4 activity.

Discussion

We identified a missense mutation in *GATA4* associated with a familial case of 46,XY DSD and congenital heart disease. This was not observed in over 800 ancestry-matched alleles, suggesting that the mutation is associated with the phenotype. Results of the functional analyses of biological activity of the mutant protein sup-

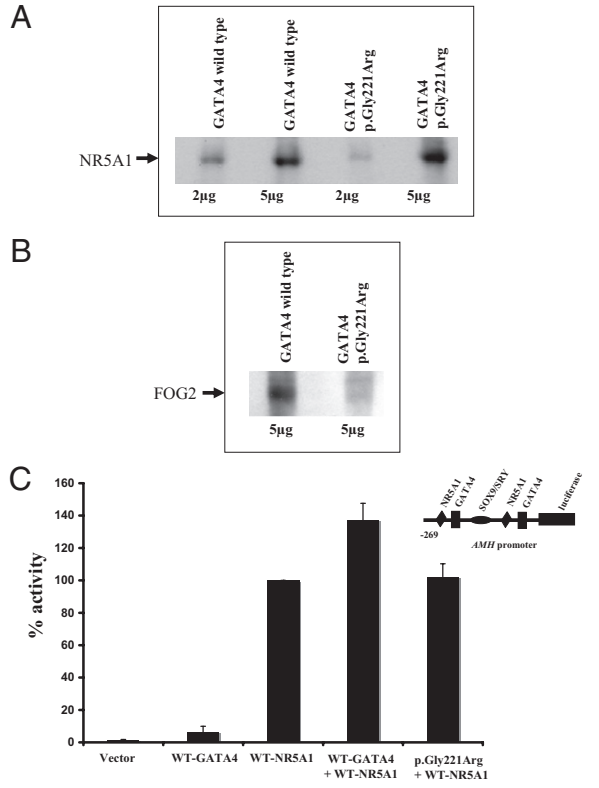

Fig. 3. (*A*) Interaction between GATA4 and NR5A1. Far Western blot analysis of the interaction between in vitro-translated WT GATA4 and GATA4 p.Gly221Arg proteins and NR5A1. Blots containing increasing concentrations of WT GATA4 (lanes 1 and 2) and GATA4 p.Gly221Arg (lanes 3 and 4) proteins were incubated with NR5A1 protein and probed by anti-NR5A1 antibody. WT and mutant GATA4 proteins can both bind to NR5A1 protein. (*B*) Interaction between GATA4 and FOG2. Far Western blot analysis of the interaction between in vitro-translated WT GATA4, the mutant GATA4 p.Gly221Arg proteins, and the WT FOG2 protein. The blot containing WT GATA4 and GATA4 p.Gly221Arg proteins was incubated with the FOG2 protein and probed by anti-FOG2 antibody, followed by secondary antibody conjugated with HRP and visualized by chemiluminescence. The blot shows that GATA4 p.Gly221Arg protein cannot interact with the FOG2 protein. (*C*) Synergy between GATA4 and NR5A1. The transcriptional synergism of WT GATA4 and GATA4 p.Gly221Arg with NR5A1 was studied using the human *AMH* promoter in HEK293-T cells. Data represent the mean ± SEM of three independent experiments, each performed in triplicate. The human *AMH* reporter construct was transfected into HEK293-T cells with either the WT or GATA4 p. Gly221Arg expression vector in the presence of NR5A1 expression vector. Results are expressed as the percentage of WT NR5A1 activity. The GATA4 p.Gly221Arg showed a statistically significant reduction ($P = 0.00652300$) in synergistic activation of the *AMH* promoter construct.

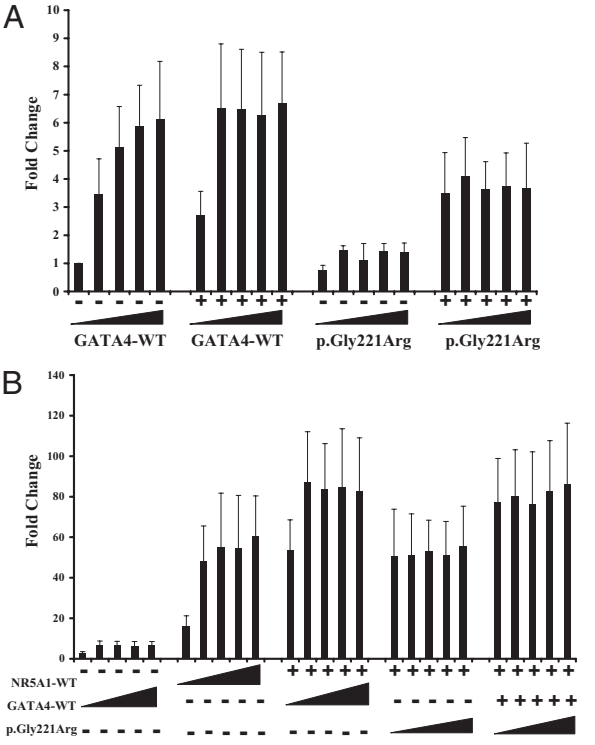

Fig. 4. (*A*) Effects of cotransfection of WT GATA4 and GATA4 p.Gly221Arg vectors on transactivation of the *AMH* promoter. The potential dominant negative effect of mutant p.Gly221Arg was studied by transfecting increasing amounts of WT or mutant GATA4 expression vector (0, 1, 2, 5, 10 ng) with 1 ng of empty vector (–) or WT vector (+) and *AMH* promoter (100 ng) in HEK293-T cells. The total amount of transfected DNA was adjusted with an empty vector. Results are expressed as the change in fold activation above the empty vector. The GATA4 p.Gly221Arg vector does not exhibit dominant negative activity on the WT GATA4 even at 10× higher concentrations. (*B*) Dominant negative effect of p.GLY221Arg on synergy between NR5A1 and WT GATA4. HEK293-T cells were transfected with *AMH* promoter (100 ng) and increasing amounts of WT GATA4 (1, 2, 3, 6, 11 ng) and NR5A1 (5, 10, 15, 20, 25 ng). A total of 15 ng of NR5A1 WT was cotransfected with increasing amounts of WT or mutant GATA4 expression vector (0, 1, 2, 5, 10 ng) and *AMH* promoter (100 ng). Increasing amounts of mutant p.Gly221Arg expression vector (0, 1, 2, 5, 10 ng) was also cotransfected with 1 ng of WT GATA4, 15 ng of NR5A1, and *AMH* promoter (100 ng). Results are expressed as the change in fold activation above the empty vector. The GATA4 p.Gly221Arg vector does not exhibit dominant negative activity on synergistic activation of the *AMH* promoter by NR5A1 and WT GATA4 even at 10× higher concentrations.

port this hypothesis. Though the p.Gly221Arg protein localized to the nucleus, it lacked DNA-binding activity and showed severely impaired transactivation of the *AHM* promoter. Although the mutant protein retained its ability to physically interact with NR5A1, it failed to synergize with NR5A1 to activate the *AMH* promoter. In addition, the GATA4 p.Gly221Arg failed to physically bind to one of its known protein cofactors, FOG2. This is remarkably similar to the observations in mice carrying a $Gata4^{ki}$ allele that encodes a mutant Gata4 p.V217G protein. The Gata4 p.V217G protein cannot interact with FOG2, and $Gata4^{ki/ki}$ XY embryos show severe impairment of testis development (15, 16).

A number of previously described *GATA4* mutations specifically associated with congenital heart defects were studied for their ability to transactivate gonadal promoters (25). In contrast to the mutation described here, these mutant proteins retained at least some DNA-binding activity and showed various degrees of transcriptional activation of the gonadal promoters. These mutant proteins also retained their ability to synergize with NR5A1 (25). This may explain the apparent absence of gonadal anomalies reported in the individuals carrying these mutations (25). Although to date, gonadal anomalies have not been reported in association with *GATA4* mutations in human, deletions of 8p encompassing the GATA4 gene are associated with genitourinary anomalies in a proportion of XY individuals (27).

GATA4 regulates the expression of multiple genes coding for hormones or components of the steroidogenic pathway during testis development and function. The phenotype observed in the three affected boys could be a primary defect in Sertoli cell function. This is supported by the observation that one of the boys had bilateral dysgenetic testes. The mutation may disrupt the expression of several Sertoli cell genes, including *AMH* and *SRY*, and we demonstrated severely impaired transactivation of the *AMH* promoter by mutant GATA4 protein. However, although the three boys in this study had very low serum AMH levels (in contrast to normal levels of testosterone), there must have been sufficient AMH produced in utero to cause regression of the Müllerian ducts. This suggests a gene dosage effect. Because the GATA4 mutation

is heterozygous, the quantity of functional GATA protein produced by the single WT allele was sufficient to produce adequate levels of AMH required for the Müllerian duct regression. This is consistent with our experimental data that do not support a dominant negative effect, whereby the mutation on one allele blocks the activity of WT protein still encoded by the normal allele, resulting in a loss-of-function phenotype (Fig. 4 *A* and *B*). We cannot exclude that the early embryonic expression of AMH may be influenced by additional factors, such as SOX9 (28). The absence of Leydig cells (case IV.3) and presence of testicular calcifications (IV.1 and IV.2) are reminiscent of the phenotype seen in mice lacking either *Amh* or its receptor, and may be attributed to deficiency in AMH signaling arising from the haploinsufficiency of functional GATA4 (29). The three affected boys had a variable clinical presentation, consistent with defects in testicular differentiation and function, and all three were raised as males.

The phenotypic variation and incomplete penetrance could be explained by genetic modifiers segregating in the family, similar to the phenotype of *Gata4*^{ki} mutant mice, which is strongly influenced by the strain background (14, 30). Genetic modifiers may include genes that encode potential protein partners of GATA4 (such as *NR5A1* and *FOG2*). In addition, GATA4 is known to recognize multiple gonadal promoters, and previous studies have indicated differential promoter sensitivity to GATA4 mutants in different cellular contexts (25). In the family we describe here, GATA4 dose-dependent effects may impact differently on separate target promoters, generating the phenotypic variability.

In 46,XX heterozygous carriers of the p.Gly221Arg mutation there was no apparent ovarian phenotype. However, GATA4 is expressed in the developing ovary as well as in adult granulosa and thecal cells (5), which suggests that either the p.Gly221Arg mutation specifically impairs testicular development or that ovarian development may be less sensitive than the testis to reduced GATA4 activity. *siGata4* transgenic mice support the latter hypothesis, where the reduction of *Gata4* expression in the testis is accompanied by a significant reduction of GATA4 downstream target genes. In the ovary, the expression of the target genes was minimally affected (31).

We show that mutation in a cofactor (GATA4) of a key protein involved in gonadal development (NR5A1) is associated with human cases of DSD and CHD. The proband is also azoospermic, suggesting that the *GATA4* mutation may also cause infertility. The proband has no evidence of heart anomalies, which suggests that other cases of 46,XY DSD and/or male infertility may also be due to mutations in GATA4. In this study, we show that GATA4 is required for human testicular development similar to its role in the mouse. This adds *GATA4* to the limited number of genetic factors known to cause human DSD.

Materials and Methods

Complete methods are described in detail in *SI Materials and Methods*.

We studied a French family with a history of 46,XY DSD. Details regarding control samples are described in *SI Materials and Methods*. We obtained written informed consent from all patients, family members, and control subjects who participated in the study. Consent forms were approved by local ethical committees.

We sequenced the coding regions of the *DMRT1*, *SRY*, *SOX9*, *NR5A1*, *NR5A2*, *WT1*, and *MAMLD1* to exclude mutations in these genes causing the gonadal anomalies (*SI Materials and Methods*). DNA from peripheral blood lymphocytes from case IV.2 underwent comparative genomic hybridization (CGH) analysis using the NimbleGen platform (HG18 CGH 385K WG Tiling v1.0; Roche) according to the manufacturer's recommendations.

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Human Male Infertility Associated with Mutations in *NR5A1* Encoding Steroidogenic Factor 1

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One in seven couples worldwide are infertile, and male factor infertility accounts for approximately 30%–50% of these cases. Although many genes are known to be essential for gametogenesis, there are surprisingly few monogenic mutations that have been conclusively demonstrated to cause human spermatogenic failure. A nuclear receptor, NR5A1 (also called steroidogenic factor 1), is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis, and it is expressed in the steroidogenic tissue of the developing and adult human gonad. Mutations of *NR5A1* have been reported in 46,XY disorders of sex development and in 46,XX primary ovarian insufficiency. To test the hypothesis that mutations in *NR5A1* cause male infertility, we sequenced *NR5A1* in 315 men with idiopathic spermatogenic failure. We identified seven men with severe spermatogenic failure who carried missense mutations in *NR5A1*. Functional studies indicated that these mutations impaired NR5A1 transactivational activity. We did not observe these mutations in more than 4000 control alleles, including the entire coding sequence of 359 normospermic men and 370 fertile male controls. *NR5A1* mutations are found in approximately 4% of men with otherwise unexplained severe spermatogenic failure.

Introduction

It is estimated that one in seven couples worldwide have problems conceiving.[1] In recent years there has been increasing concern about a possible decline in reproductive health, and this trend is paralleled by an increasing demand for infertility treatments. As many as 8% of children in some Western countries are born as a result of assisted reproductive techniques.[2] Sperm counts in several European countries are declining, and in Denmark 20% of healthy young adult males have sperm concentrations below the World Health Organization reference level of 20×10^6 sperm/ml.[1,3] In the majority of cases, the underlying cause of male infertility is unknown. Familial clustering of male subfertility as well as families with multiple infertile or subfertile men, in whom an autosomal-recessive or -dominant mutation with sex-limited expression is likely to be present, indicates a genetic contribution to spermatogenic failure.[4–6] A chromosomal anomaly is carried by 5% of all infertile men (such as 47,XXY Klinefelter syndrome), and microdeletions of the long arm of the Y chromosome (MIM 415000) are present in 10% of azoospermic or severely oligozoospermic ($< 1 \times 10^6$ sperm/ml) men.[7] Although rodent studies indicate that multiple genes have the potential to cause male infertility, only a few single-gene defects that cause male infertility have been identified in humans. These include *AURKC* (MIM 603495) mutations associated with large-headed, multiflagellar polyploid spermatozoa (MIM 243060), *SPATA16* (MIM 609856) mutations associated with globozoospermia (MIM 102530), *CATSPER1* (MIM 606389) mutations associated with recessive male infertility (MIM 612997), and mutations of the dynein genes that encode proteins of the axonemal dynein cluster (*DNAH1* [MIM 603332], *DNAH5* [MIM 603335], *DNAH11* [MIM 603339]) and are associated with asthenozoospermia.[8] However, the collective prevalence of these mutations is extremely low.

NR5A1 (MIM 184757), a member of the nuclear receptor superfamily, is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis.[9,10] NR5A1, also called steroidogenic factor-1, consists of a DNA-binding domain (DBD) including two zinc fingers, a flexible hinge region, a ligand-binding domain (LBD), and two activation function domains: AF-1 and AF-2.[11–13] NR5A1 binds DNA as a monomer, and it is expressed in Sertoli and Leydig cells of the developing testis and in Sertoli cells of the prepubertal and adult testis, as well as in multiple cell types in the fetal, postnatal, prepubertal, and mature ovary.[14–16] In mammalian testis determination and differentiation, NR5A1 is a positive regulator of SOX9 (Sry-box 9) and Anti-Müllerian Hormone (AMH).[17,18] NR5A1 also modulates the expression of many factors involved in cholesterol mobilization and steroid hormone biosynthesis, including HMG-CoA synthase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), and several cytochrome P450 steroid

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hydroxylase (CYP) enzymes.[17] Consistent with its key role in gonadal development, *NR5A1* mutations are associated with a wide spectrum of phenotypes, including 46,XY partial and complete gonadal dysgenesis with or without adrenal failure (MIM 612965), penoscrotal hypospadias, micropenis with anorchidia, and 46,XX primary ovarian insufficiency (POI [MIM 612964]).[17,19]

Here, we demonstrate that heterozygous mutations in *NR5A1* are also associated with severe spermatogenic failure in otherwise healthy men. In an analysis of 315 men seeking infertility treatment because of spermatogenic failure, we identified heterozygous missense mutations in seven men, each mutation located in the hinge region and proximal LBD of the protein. Each of the mutant proteins fails to transactivate gonadal promoters optimally. Our data increase the spectrum of phenotypes that are associated with mutations in *NR5A1*.

Subjects and Methods

Patient and Control Populations

The study was approved by the Institut Pasteur institutional review board (RBM 2003/8). We obtained written informed consent from all patients, family members, and control subjects who participated in the study. A total of 315 men who had unexplained reduced sperm counts and were seeking infertility treatment were included in this study. All men were recruited from one infertility clinic in Paris. The men were of mixed ancestry, and they are representative of the local Parisian population. Patient ancestry was determined by self reporting, based on responses to a personal questionnaire, which asked questions pertaining to the birthplace, languages, and ethnicity of the participants, their parents, and their grandparents. Infertile men with known causes of infertility, including chromosome anomalies, Y chromosome microdeletions, cryptorchidism, hypospadias, occupational hazards, varicocele, and lifestyle factors, were excluded from this study. Control samples were obtained from the HGDP-CEPH panel, comprising 1064 DNA samples from 52 worldwide populations. Additional control samples consisted of 140 French men, 89 men of West African origin (kindly provided by Dr. Anavaj Sakuntabhai of the Institut Pasteur, Paris), and 96 men of North African origin. Although these men are healthy, their fertility and semen quality is unknown. To investigate the degree of rare genetic variation in *NR5A1*, we sequenced the entire open reading frame of *NR5A1* in DNA from a panel of 370 fertile men (father of at least two children) and 359 men with normal semen parameters (European descent n = 331, North African descent n = 140, West African descent n = 63, Indian descent n = 156, East Asian descent n = 30, other descent n = 9).

Mutational Analysis of *NR5A1*

The coding exons of *NR5A1* (exons 2–7; NM_004959.4) were amplified from DNA extracted via conventional techniques from peripheral-blood lymphocytes of each individual and sequenced in accordance with protocols described elsewhere.[19]

Site-Directed Mutagenesis

NR5A1 expression vectors containing the p.Pro131Leu, p.Arg191Cys, p.Asp238Asn, and p.Gly212Ser variants were generated by site-directed mutagenesis (QuikChange, Stratagene) with the use of wild-type (WT) human *NR5A1* cDNA in a pCMX expression vector as a template.[19] The entire coding sequence of all mutant plasmids was confirmed by direct sequencing prior to functional studies.

Transient Gene Expression Assays

Transient gene expression assays for the assessment of NR5A1 function were performed in 96-well plates (TPP) with the use of either human embryonic kidney (HEK) 293T cells or a mouse embryonic stem cell line (E14), Fugene 6 transfection reagent (Roche no. 1 814 443), and a Dual-Luciferase reporter assay system (Promega) with pRLSV40 Renilla luciferase (Promega) expression as a marker of transfection efficiency. pCMX_WT or mutant NR5A1 expression vectors (10 ng/well) were cotransfected into HEK293T cells with reporters containing NR5A1 (SF1) responsive minimal promoters (murine *Cyp11a1*, human *AMH*) (10 ng/well).[20,21] Cells were lysed 48 hr later, and luciferase assays were performed with the use of a FLUOstar Optima fluorescence microplate reader (BMG Labtech). All data were standardized for Renilla activity. Results are shown as the mean ± SEM of three independent experiments, each performed in triplicate. A previously described inactivating mutation of NR5A1, p.Gly35Glu, was included as control in the transactivation studies.[21]

Cellular Localization Studies

WT *NR5A1* cDNA was cloned into a pAcGFP-C1 vector (Clontech) to allow expression of GFP-tagged NR5A1. *NR5A1* mutations were introduced by site-directed mutagenesis. Plasmids (0.8 μg/well) were transfected into tsa201 cells with the use of Lipofectamine 2000 (Invitrogen), and images were obtained 24 hr later with a Zeiss Axioskop microscope and camera.

In Vitro Protein Expression

An in vitro rabbit reticulocyte-coupled transcription/translation system (TNT Quick Coupled Transcription/Translation System, Promega) was used to express proteins from the vector constructs. The reactions were performed according to the manufacturer's instructions. In brief, biotin-labeled protein was expressed by incubation of 1 μg of vector DNA with reticulocyte lysate, amino acid mixture, RNasin, T7 RNA polymerase, and Transcend Biotin-Lysyl-tRNA (Promega) in a final volume of 25 μl at 30°C for 90 min.

Table 1. Mutations in *NR5A1* Associated with Spermatogenic Failure

Patient	Age at Investigation	Ethnic Origin	Karyotype	*NR5A1* Mutation	Sperm Count (10^6/ml) N: > 20 × 10^6/ml	FSH (IU/l) N: 1.0–10.5 IU/l	LH (IU/l) N: 0.7–8.0 IU/l	Testosterone (ng/ml) N: 3.0–10 ng/ml	Inhibin B (pg/ml) N: 80–400 pg/ml
1	42	Congolese	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	0	72	34.3	0.49	<15
2	37	Congolese	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	0	NA	NA	NA	NA
3	29 and 31[b]	Tunisian	46,XY	p.Gly123Ala (c.368G>C)/ p.Pro129Leu (c.386C>T)[a]	12 and 6[c]	5.1	4.3	5	74
4	41	Sri Lankan[d]	46,XY	p.Pro131Leu (c.392C>T)	0	NA	NA	NA	NA
5	25	Congolese	46XY	p.Arg191Cys (c.571C>T)	0.3	18.8	10.7	5.7	<15
6	37	French-Vietnamese	46XY	p.Gly212Ser (c.634G>A)	0.8	NA	NA	NA	NA
7	41	Egyptian	46,XY	p.Asp238Asn (c.712G>A)	0.7	15.1	6	3	31

NA, not available.
[a] Mutation was previously reported as associated with POI.
[b] Patient was evaluated over a 2 yr period.
[c] Semen quality decreased over a 2 yr period. There was also a reduction in sperm motility and viability.
[d] Mutation also observed in a woman with POI who is of Tamil origin (unpublished data).

Sumoylation of Mutated Proteins

In vitro sumoylation assays were carried out by incubating in vitro translated pCMXSF1 or p.Asp238Asn vectors with recombinant Aos1/Uba2 (370 nM), Ubc9 (630 nM), and SUMO (7 μM) in 30 mM Tris, 5 mM ATP, 10 mM $MgCl_2$, pH 7.5, at 33°C as previously described.[22]

Results

NR5A1 Mutations Were Identified in Infertile Men

In a screen of 315 men with unexplained spermatogenic failure who sought infertility treatment, we found seven heterozygous mutations in *NR5A1* by direct sequencing (Table 1). The seven men carrying *NR5A1* mutations did not report any other members of the family with phenotypes known to be associated with *NR5A1* mutations, such as POI or 46,XY disorder of sex development (DSD), and there was no evidence of undervirilization, nor were there signs of adrenal insufficiency. No other family members were available for genetic analysis, so it is unknown whether the mutations are de novo. However, three men of African origin carried a double *NR5A1* mutation (p.Gly123Ala/p.Pro129Leu; NP_004950) that we have previously reported as being associated with POI in a girl of African origin, suggesting that this is probably a founder mutation.[19] One man, who carried the p.Gly123Ala/p.Pro129Leu double mutation, had a progressive loss of germ cell quantity and quality over a 2 yr period (Table 1). With one exception, *NR5A1* mutations were associated with severe spermatogenic failure (Table 2). We did not observe mutations in men with mild oligozoospermia. These mutations were not observed in over 2100 control samples (4200 alleles), and no rare allelic variants were found after analysis of the entire coding region of *NR5A1* in 370 fertile (father of at least two children) or 359 normospermic men (Table 2). This indicates that these mutations are pathogenic. All mutations fall within the evolutionarily conserved hinge region (amino acids 95–225), or the proximal portion of the LBD of NR5A1 (Figure 1).

NR5A1 Mutations Are Associated with Altered Hormonal Profile and Gonad Histology

Hormonal data were available for four of the men carrying *NR5A1* mutations (Table 1). Testosterone levels were at the lower limit of the normal range in subject 7 and below the normal range in subject 1. Serum levels of inhibin B, a marker of spermatogenesis and a predictor of the presence of testicular sperm in men with nonobstructive

Table 2. Frequency of *NR5A1* Mutations and Associated Phenotypes

Phenotype	No. of Individuals	No. of Individuals with Mutation in *NR5A1*
Azoospermia or cryptozoospermia	103	4 (3.9%)
Severe oligozoospermia or OATs < 1 × 10^6/ml	46	2 (4.3%)
Moderate oligozoospermia or OATs 1–10 × 10^6/ml	50	1 (2%)
Mild oligozoospermia or OATs 10–20 × 10^6/ml	116	0
Fertile[a]	370	0
Normospermic[a]	359	0

[a] The entire open reading frame of *NR5A1* was sequenced in each individual.

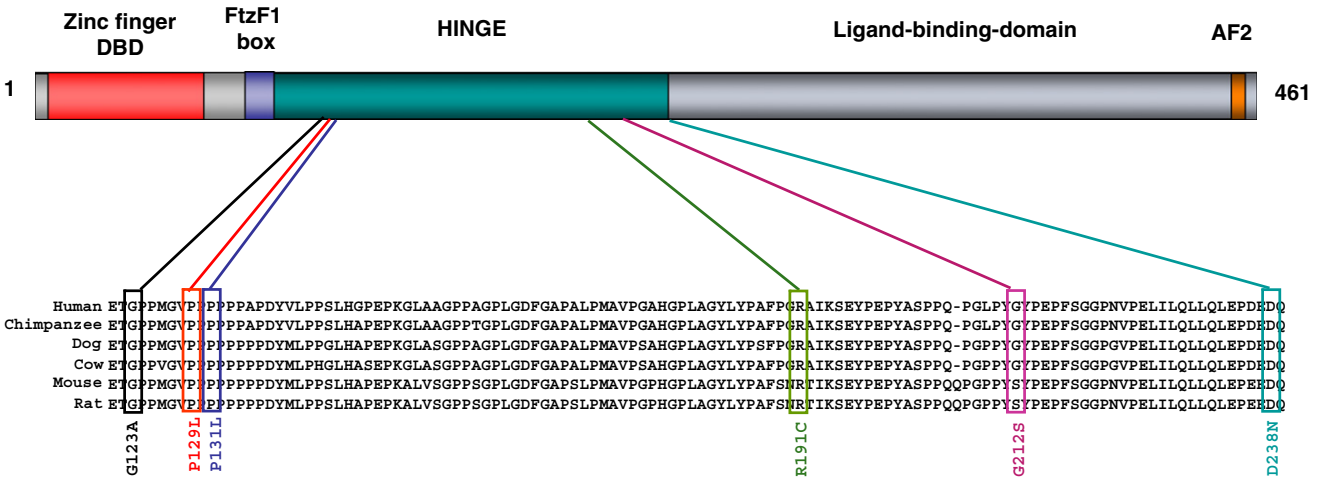

Figure 1. Distribution of NR5A1 Mutations Associated with Spermatogenic Failure in Relation to the Protein
The functional domains of the NR5A1 protein are shown. The DNA-binding domain containing two zinc-finger motifs is indicated. The FtzF1 box stabilizes protein binding to DNA. The hinge region is important for stabilizing the ligand-binding domain and interacts with other proteins that control NR5A1 transcriptional activity. The AF2 domain recruits cofactors necessary for NR5A1 transactivating activity. The position of the amino acid change and its evolutionary conservation are shown for each of the mutations identified.

azoospermia, were low in all four men. Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were elevated above the normal values in cases 5 and 1, whereas case 7 had elevated serum FSH and LH levels within the normal range. Gonadal histology was available for case 1, who carries the p.Gly123Ala/p.Pro129Leu double mutation (Figure 2). This showed a hypoplastic testis with few germ cells and areas of marked fibrosis and hyalinization.

NR5A1 Mutations Do Not Affect Nuclear Localization but Alter the Transactivation Ability of the Protein

To assess the impact of the NR5A1 mutations on nuclear localization, we generated WT and mutant GFP-NR5A1 constructs by cloning WT *NR5A1* cDNA in frame into a pAcGFP-C1 vector to produce a fusion protein of NR5A1 with a monomeric green fluorescent protein (GFP) tag at its amino-terminal end. Mutant pAcGFP-C1-NR5A1 vectors were generated by site-directed mutagenesis, with the WT construct used as a template. The cellular localization of both WT and mutant GFP-NR5A1 fusion proteins (green), generated and expressed in tsa201 cells with the use of the pAcGFP-C1 vector, showed strong nuclear localization with relative nucleolar exclusion and very occasional nuclear subfoci (Figure 3).

To assess the functional properties of NR5A1, we performed site-directed mutagenesis by using WT human *NR5A1* cDNA in a pCMX expression vector as a template.

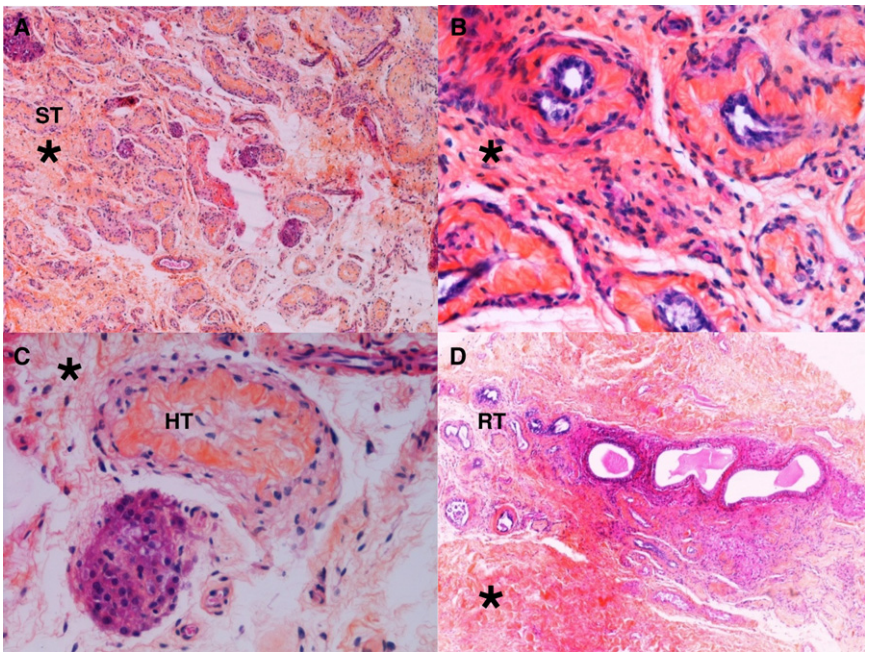

Figure 2. Gonadal Histology of Subject 1
Photomicrographs demonstrating abnormal testicular histology showing areas of interstitial fibrosis (A–D) and hyalinization (C), with scattered residual abnormal seminiferous tubules containing occasional germ cells but no normal spermatogenesis. Within the fibrous areas, residual tubular structures are present (D). No normal testicular tissue is present. (ST, seminiferous tubules; *, interstitial fibrosis; HT, hyalinised tubule; RT, residual tubular structures).

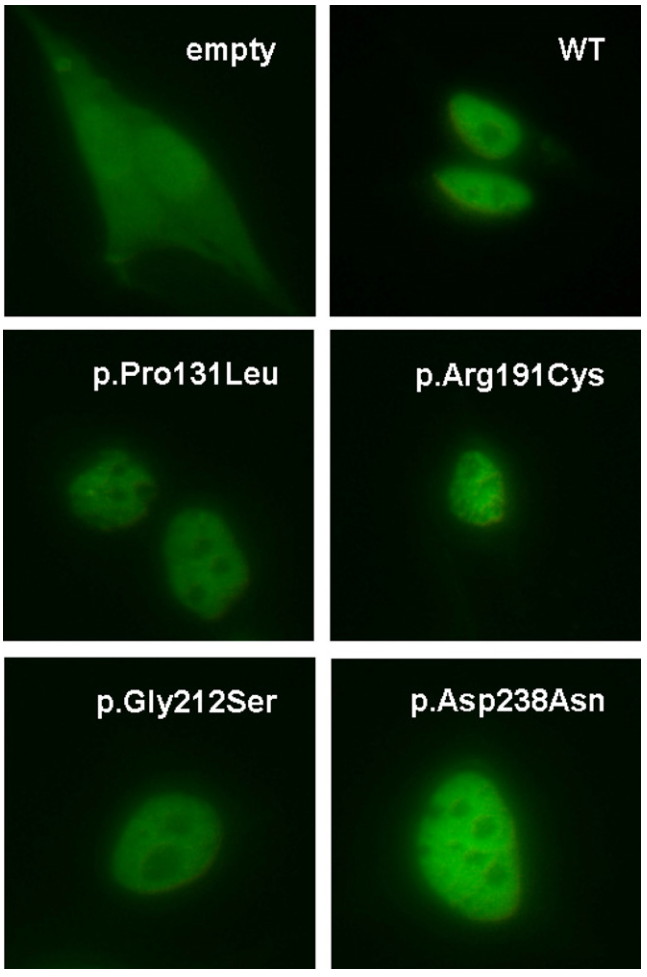

Figure 3. Cellular Localization of NR5A1 Mutants
Cellular localization of GFP-SF1 fusion proteins (green), generated and expressed in tsa201 cells with the use of a pAcGFP-C1 vector. WT NR5A1 shows strong nuclear localization, with relative nucleolar exclusion and very occasional nuclear subfoci. An expression and localization pattern similar to that of the WT was seen for all the other mutant proteins.

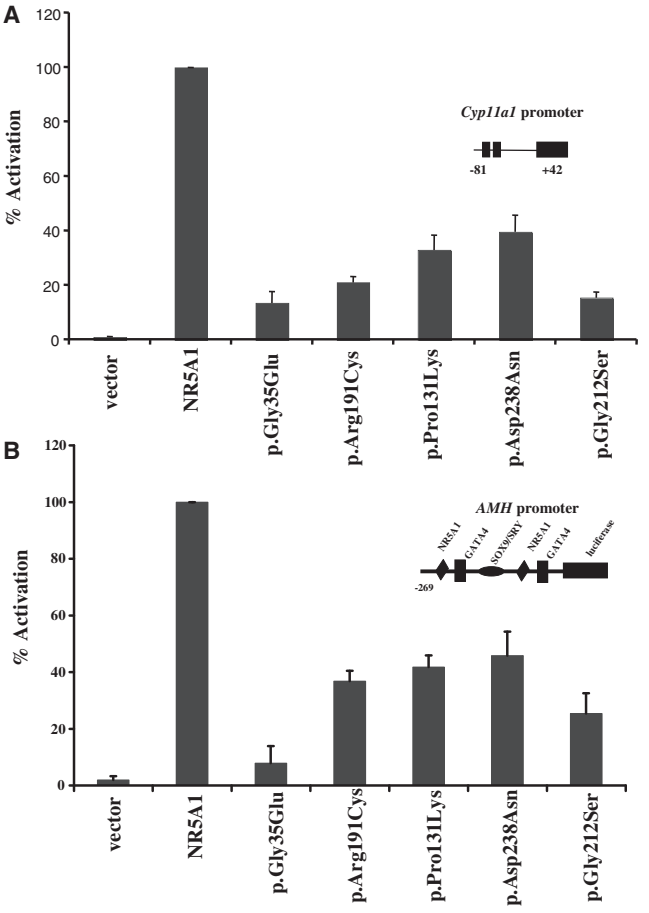

Figure 4. Assays of NR5A1 Transcriptional Activity
The transcriptional activity of WT NR5A1 and variants associated with male infertility was studied with the use of *Cyp11a1* (A) and *AMH* (B) promoters in HEK293T cells. A previously described inactivating mutation of NR5A1, p.Gly35Glu, was included as a control in the transactivation studies. Results are expressed as a percentage of WT NR5A1 activity, which is considered to be 100%. Data represent the mean of three independent experiments, each performed in triplicate. The T bars represent the SEM.

We have previously demonstrated that the NR5A1 p.Pro129Leu variant lacks transcriptional activity, and this mutant protein is associated with 46,XX POI.[19] The other mutant proteins had altered biological activity in cotransfection luciferase assays driven by NR5A1-dependent gonadal promoters. A quantitative reduction in the transactivation of both the *Cyp11a1* (encoding P450scc) promoter and the *AMH* (encoding anti-Müllerian hormone) promoter was observed in transactivation assays of the mutations p.Pro131Leu, p.Arg191Cys, p.Gly212Ser, and p.Asp238Asn (NP_004950) using HEK293T cells (Figures 4A and 4B). Similar results were obtained in transient gene expression assays using murine E14 embryonic stem cells (data available on request). These in vitro functional assays demonstrated that each mutation may lead to a functional disturbance of the NR5A1 protein and may affect the regulation of its downstream target genes during gonadal development and function.

NR5A1 Mutation p.Asp238Asn Does Not Affect Sumoylation of NR5A1

The NR5A1 p.Asp238Asn mutation was analyzed for its ability to undergo sumoylation because it lies immediately carboxy terminal to a putative SUMO-binding motif. No difference was observed between the efficiency of pCMX-SF1 and pCMX-Asp238Asn for undergoing in vitro sumoylation (data available upon request).

Discussion

In this study, we provide evidence that mutations in *NR5A1* (encoding steroidogenic factor 1) are associated with unexplained severe spermatogenetic failure in otherwise healthy men. This considerably broadens the range of phenotypes associated with mutations in *NR5A1*, which to date have been reported only in association with more severe forms of gonadal dysgenesis or with significant genital anomalies

such as penoscrotal hypospadias, anorchia, or undescended testes.[17] These data therefore support the hypothesis of Skakkebaek and coworkers that a subset of men with spermatogenic failure have a mild form of testicular dysgenesis syndrome.[1,23] Although male factor infertility is the primary presenting feature leading to medical evaluation in these cases, our data show that this subset of men with severe azoospermia may also be at risk of endocrine dysfunction and failing testosterone with increasing age. It is well established that approximately 12%–15% of men with idiopathic spermatogenic failure have reduced serum testosterone levels and elevated LH levels as compared to the normal range.[24] It has been proposed that some of these men may have mild forms of testicular dysgenesis.[24] Our data suggest that those individuals with *NR5A1* mutations may represent part of this group.

The mutations in NR5A1 reported here were all missense mutations in the hinge region or proximal ligand-binding domain of the protein. We found the p.Gly123Ala/p.Pro129Leu double mutation in three individuals of Central or North African ancestry, and we have described this change previously in a West African girl with POI, suggesting that this mutation may be present at low levels in the general population.[19] The transmission of the mutation may be explained by a progressive loss of gonadal function over time, so that fecundity is achieved in early adulthood before the development of spermatogenic failure. In this study, two men who carried this mutation presented with azoospermia at 37 and 42 yrs of age. Individual 3, who also carried this mutation, showed a progressive decline in both sperm quantity and quality over a 2 yr period, from 29 yrs to 31 yrs of age. This may represent a progressive aging phenomenon or may possibly represent different expression of the phenotype as a result of other genetic or environmental modifiers. The absence of this allelic variant in the control population suggests that this is not a frequent genetic alteration. Furthermore, the absence of any changes in *NR5A1* after direct sequencing of more than 600 fertile or normospermic men suggests that rare allelic variants in this gene are not common, other than the well-described p.Gly146Ala polymorphism (rs1110061).

A number of molecular mechanisms may explain the spermatogenic failure associated with *NR5A1* mutations. In the *Nr5a1* Leydig cell-specific knockout, mice had hypoplastic testes in which the lumens of the seminiferous tubules failed to open and spermatogonia never developed into mature sperm.[25] These mice also showed reduced expression of two key genes in testosterone biosynthesis, *Cyp11a* and *StAR*.[25] In a study of azoospermic patients, expression levels of *NR5A1* in gonadal tissue correlated positively with serum testosterone concentrations, suggesting a direct connection between these two factors.[26] Alternatively, impairment of reproductive function could result, in part at least, from anomalies of the anterior pituitary. Mice that lack *Nr5a1* specifically in their pituitary have reduced levels of LH and FSH.[27] These mice show marked hypogonadism with a reduction in testis volume, a decreased number of Leydig cells, and an absence of mature spermatids, resulting in infertility.[27] However, our data show normal or elevated serum FSH and LH levels together with a normal or low testosterone level in men carrying an *NR5A1* mutation that suggests a predominant testicular phenomenon.

We show that the NR5A1 mutants associated with male infertility show impaired activation of two of the NR5A1 target genes, *AMH* and *Cyp11a1*. Several molecular mechanisms could explain the germ cell loss associated with these mutations. The mutations fall within the hinge region (amino acids 95–225) and proximal portion of the LBD, and a number of physical interactions and functional activities have been mapped to this portion of the protein.[12] Phosphorylation of Ser 203 in the hinge region enhances the interaction of GRIP1 and SMRT with the AF1 and AF2 regions of NR5A1, whereas sumoylation of lysines within the hinge region increases interactions with DEAD box proteins and results in transcriptional repression.[28,29] NR5A1 stimulation of CYP17A1 expression is augmented by a direct physical interaction with the protein translin.[30] This interaction is mediated through amino acids 170–225 of the hinge region of NR5A1.[30] Modulation of NR5A1 transcriptional activation via the hinge region has also been described for the coactivator SRC-1, which potentiates the activity of SF-1 by utilizing the highly-conserved AF-2 hexamer at the C terminus of the protein and a proximal interaction domain at residues 226–230.[16] Finally, in vitro studies have suggested that NR5A1 receptor phosphorylation may be modulated by the herbicide atrazine, leading to disruption of NR5A1-related gene networks and potential alterations in endocrine development and function.[31] The clustering of the mutations in a specific region of the molecule could suggest a common mechanism leading to germ cell loss, or even altered sensitivity to environmental disruptors, but additional studies of larger cohorts of infertile men are required to see whether such a genotype-phenotype correlation is robust.

We conclude that approximately 4% of men with otherwise unexplained severe spermatogenic failure carry mutations in *NR5A1*. The data also suggest that some forms of male infertility may be an indicator of mild testicular dysgenesis, underlining a need for careful clinical investigation of men presenting with infertility and incongruous testosterone and gonadotropin levels.

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Web Resources

The URLs for data presented herein are as follows:

HGDP-CEPH Human Genome Diversity Cell Line Panel, http://www.cephb.fr/HGDP-CEPH-Panel
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

Gene–Environment Interactions in Panic Disorder and CO_2 Sensitivity: Effects of Events Occurring Early in Life

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Heterogeneous life events (LE) precede the onset of—and potentially increase the susceptibility to—panic disorder (PD). It remains unknown whether LE can act as moderators in the context of gene-by-environment interactions (G×E) that alter the susceptibility to PD and the related trait of CO_2 sensitivity, nor it is known whether such moderation may depend on occurrence of events at different epochs in life. In 712 general population twins we analyzed by Maximum Likelihood analyses of ordinal data whether life (major- and stressful) events moderate the genetic risk for PD and CO_2 sensitivity, as indexed by the 35% CO_2/65% O_2 challenge. For CO_2 sensitivity, best-fitting models encompassed both additive and interactional effects that increased linearly with the cumulative number and severity (SEV) of events in lifetime. By analyzing the moderation effect of cumulative SEV separately for events that had occurred in adulthood (between age 18 and 37) or during childhood–adolescence (before the 18th birthday), we found evidence of G×E only within the childhood–adolescence window of risk, although twins had rated the childhood–adolescence events as significantly ($P = 0.001$) less severe than those having occurred during adulthood. For PD, all interactional terms could be dropped without significant worsening of the models' fit. Consistently with a diathesis-stress model, LE appear to act as moderators of the genetic variance for CO_2 sensitivity. Childhood–adolescence appears to constitute a sensitive period to the action of events that concur to alter the susceptibility to this panic-related trait. © 2010 Wiley-Liss, Inc.

Key words: genetics; childhood; twins; endophenotype

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INTRODUCTION

Panic disorder (PD) is a heritable multifactorial condition, with genetic influences accounting for 30–40% of phenotypic variation, and both shared and idiosyncratic environmental factors explaining the rest of variance [Kendler et al., 1993, 2001; Battaglia et al., 2008b]. Heterogeneous life events (LE) and adversities, including interpersonal conflicts and physical illnesses, appear to affect the individual susceptibility to PD [Manfro et al., 1996], according to both clinical [Faravelli and Pallanti, 1989] and epidemiological [Faravelli et al., 2007] studies. In spite of such apparently consistent association, the mechanisms that link the occurrence of LE to the manifestations of PD remain largely unknown. Major confounders of the relationships that tie LE to the manifestation of PD derive from the methods of patients' selection [Kendler et al., 1998] and

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unreliable recollection [Faravelli and Pallanti, 1989; Manfro et al., 1996], implying that subjects who have experienced symptoms would strive more than healthy controls to recall the occurrence of LE, as a posteriori explanations for their psychopathology [Kendler et al., 1998]. Endophenotypes [Gottesman and Gould, 2003] that share part of the determinants of susceptibility with a disorder, but are at least partially independent of the clinical syndrome's manifestation, can be employed to investigate pathogenetic mechanisms in unaffected subjects, and circumvent some of these confounders [Gottesman and Gould, 2003; Gould and Gottesman, 2006]. A putative endophenotype of DSM-IV PD is the anxious response to CO_2 stimulation [Smoller and Tsuang, 1998; Fyer and Weissman, 1999] that covaries with PD largely because of shared genetic determinants [Battaglia et al., 2008b]. We [Ogliari et al., 2010] recently found that in general population twin subjects, the anxious response to CO_2 stimulation is affected by several of the same antecedents/events that have been associated to naturally occurring panic attacks and PD. This raises the possibility that LE moderate a proportion of genetic liability to PD and/or to the related trait of CO_2 sensitivity through gene-by-environment interaction (G×E) mechanisms [Rutter et al., 2006].

G×E mechanisms assume that the genetic variance changes as a function of environmental exposure [Purcell, 2002; Rutter et al., 2006]. While G×E effects are biologically sensitive and commonly found in experimental organisms [Mather and Jinks, 1982], quantitative biometric studies of human behavioral disorders have often led to question the veracity and/or the importance of G×E effects [Eaves et al., 1977; Eaves, 2006]. Spurious interactions can emerge from scale of measurement problems [Eaves, 2006], and—when present—the contributions of interactions are typically smaller than those of the main effects [Mather and Jinks, 1982]. On the other hand, recent empirical [Caspi et al., 2003; Kendler et al., 2005; Uher and McGuffin, 2010; Vinkhuyzen et al., 2010] and conceptual [Caspi and Moffitt, 2006; Rutter et al., 2006] work substantiates G×E effects in psychopathology. Recent reviews also emphasize how different strategies to investigate G×E, such as the "theoretical" (quantitative-biometric)—versus "measured" (based on specified genetic polymorphisms and identified environmental hazardous factors) approach, may impact upon findings [Rutter et al., 2006].

Regardless of their technical standpoints, many authors [Hunter, 2005; Eaves, 2006; Moffitt et al., 2006; Khoury and Wacholder, 2009] agree that one bottleneck—both conceptual and methodological—for adopting/discarding claims in this growing field lies in the ultimate biological value and meaning of any new G×E findings. In this light, the adoption of endophenotypes in G×E research [Caspi and Moffitt, 2006; Battaglia et al., 2008a] has two main advantages. First, G×E investigations could shed light upon processes, pathways and mechanisms of illness that the classical, additive-linear model of risk might miss [Rutter et al., 2006]. By adopting endophenotypic physiological measures that map functional traits en route between multiple elements of liability and a related clinical phenotype, G×E studies could probe-specific pathogenetic junctures, or some neurofunctional components of an illness. Thus, inasmuch as endophenotypes map pathways that are part—but not necessarily the entirety—of an etiopathogenetic process toward a fully manifest disorder [Caspi and Moffitt, 2006; Gould and Gottesman, 2006], they could reveal biologically meaningful G×E effects that may go undetected when the object of investigation is the broader, and more heterogeneous, construct of a clinical disorder. Second, valid endophenotypes yield by definition [Gottesman and Gould, 2003] deviant scores amongst individuals who are at heightened risk without bearing measurable psychopathology (such as the unaffected first-degree relatives of affected individuals) [van Beek and Griez, 2000; Coryell et al., 2001, 2006]. If it is found that G×E mechanisms affect an endophenotypic measure, then a dimensionally distributed function of risk can be inferred in the general population [Rutter and Plomin, 2009], and pave the way toward characterizations of vulnerability beyond the affected/unaffected dichotomy, via the application of neurofunctional techniques. Quantitative biometric investigations of G×E based on endophenotypes thus appear to lye midway between the "classic" biometric quantitative approach to purely behavioral phenotypes, and the "hypothesis-driven" molecular-genetic approach, which typically and sometimes forcibly, focuses a small number of identified genetic polymorphisms and specified factors of environmental hazard.

While both heterogeneous environmental adversities and genetic factors have been shown to influence the individual susceptibility to PD, there are no published studies of G×E on PD [Maron et al., 2010] and CO_2 sensitivity. The only possible exception is a questionnaire-based study showing an increase of genetic variance for somatic/panic anxiety symptoms across higher levels of self-assessed negative events in twins with a mean age of 15 years [Lau et al., 2007], an age, however, at which PD manifests relatively rarely.

In a sample of young adult twins, we examined two principal questions. First, we investigated whether LE can act as moderators in G×E that alter the individual susceptibility to PD and the related endophenotypic trait of CO_2 sensitivity. Second, since the effects of childhood and adolescence adversities can persist beyond the developmental years [Kessler et al., 1997; Zlotnick et al., 2008; Green et al., 2010], we investigated whether G×E effects may depend on the occurrence of LE during different lifetime windows of risk, such as childhood and adolescence as opposed to adulthood.

MATERIALS AND METHODS

Sample

The sample collection methods have been fully detailed elsewhere [Battaglia et al., 2007, 2008b, 2009; Ogliari et al., 2010]. Briefly, from 2002 to 2004 we consecutively recruited 712 subjects (346 complete pairs: 169 monozygotic (MZ) and 177 dizygotic (DZ), plus 20 single twins) from the Norwegian Twin Study on the Genetics of Personality and Mental Health cohort [Harris et al., 2002]: 64% were women, the mean age at interview was 30.95 years, range 22–37, standard deviation (SD) 3.6 years, and the prevalence of lifetime *DSM-IV* PD was 6.6% [Battaglia et al., 2009]. We gathered information on CO_2 reactivity, extensive personal history on the occurrence of LE, and on lifetime DSM-IV diagnoses by the Composite International Diagnostic Interview (CIDI) [Robins et al., 1988] administered by trained interviewers [Harris et al., 2002; Battaglia et al., 2008b].

Sensitivity to CO_2 was assessed by a 35% CO_2/65% O_2 single-breath challenge [Griez et al., 1990] followed by air (as placebo), which discriminates patients with PD from controls and patients with other mental disorders [Battaglia and Perna, 1995]. Subjects provided post-CO_2 ratings of subjective anxiety with a Visual Analogue Scale for Anxiety [Wolpe, 1973] (VASA, from: 0 no anxiety at all to 100, the worst anxiety imaginable) obtained immediately after inhalation, and evaluated as the percentage of maximum increment or decrement possible from the VASA pre-test score (Δ% VASA).

After complete description and screening of exclusion criteria, participant signed an informed consent [Battaglia et al., 2007].

Record and Parametrization of Life Events

In the interval between the two gas mixture (35% CO_2/65% O_2 and air) inhalations two interviewers blinded to the participant's diagnosis investigated the subject's lifetime history of DSM-IV diagnoses, PD antecedents, and occurrence of LE by a structured interview [Ogliari et al., 2010]. The interview covered five antecedents'/risk factors' areas, selected after extensive review of the literature [Kendler et al., 1992; Battaglia et al., 1995; Manfro et al., 1996; Bouwer and Stein, 1997; Horesh et al., 1997; Aschenbrand et al., 2003] namely: (a) childhood parental loss/separation (CPL); (b) symptoms of DSM-IV childhood separation anxiety disorder (SAD); (c) events of suffocative nature; (d) major LE; and (e) stressful events. Events were counted only once for these five areas, so that, e.g., the death of a parent during childhood was counted within the CPL area, but not among the stressful events. The impacts of CPL, suffocative events and SAD upon individual susceptibility to DSM-IV PD and/or CO_2 sensitivity have already been analyzed in this sample, with purely additive (i.e., non interactional) models providing the most parsimonious and best -fitting explanation for phenotypic variance and covariance, as previously reported [Battaglia et al., 2009; Ogliari et al., 2010]. Thus, here we focus on the moderating role of those putative risk factors whose G×E effects: (a) have not yet been analyzed in this sample and (b) could be characterized in terms of occurrence, severity and age at incidence during the interview. These were: The major LE and the stressful LE from the interview on risk factors, which are here cumulatively counted and referred to as "life events" for this study.

Whenever a participant endorsed the occurrence of major- or stressful LE, in addition to the age at first exposure, the interviewer recorded the proband's rating of subjective severity on a likert four point scale (from 0 = not stressful at all to 3 = extremely stressful). A complete list of the LE employed in the G×E modeling is reported in Table I.

We derived two measures of LE: One was the cumulative (from birth to day of experiment) number (NUM) of events endorsed by probands during the interview, and the other was the cumulative severity (SEV) of these same LE. While obviously related, these two measures yield relatively different information: For the NUM variable, all LE have equal weight regardless of the differential emotional impact they may have exerted on a proband, whereas the SEV variable is a sum of the subjective cumulative severity of all possible LE a proband had been exposed to. Thus, while NUM capitalizes solely upon the quantity of LE, SEV constitutes a more comprehensive measure, in that it is a cumulative estimate encompassing both the number and the perceived severity of LE during a subject's lifetime.

TABLE I. List of the Events Employed to Build the NUM- and SEV Scales of Life Events

Major LE	◯Life-threatening illnesses
	◯Life-threatening accidents
	◯Being exposed to physical abuse
	◯Being exposed to physical assaults
	◯Being exposed to other severe threats or to one's life/integrity
Stressful events	◯Separation from spouse/partner
	◯Other marital problems
	◯Broken relationship with your mate
	◯Separation from close friend
	◯Separation from loved one
	◯Jail term
	◯Disability retirement
	◯Major financial problems
	◯Marital reconciliation
	◯Getting a new boy/girl friend into a stable relationship
	◯Start living together with your mate
	◯Gain a new family member coming to live with you
	◯Having a child
	◯Difficulties in one's sexual life
	◯Major business readjustments
	◯Begin/end school
	◯Major changes in one or more of the following
	Work hours
	School
	Recreation
	Residence
	Social/church activities

Both NUM and SEV were z-transformed for analyses. For participants who had developed DSM-IV PD/panic attacks, the association with adversities was modeled only for NUM and SEV of LE that had occurred before onset of PD/panic attacks. Moreover, there is growing interest in investigating the effects of those environmental agents of risk that by striking in childhood and adolescence affect physical and mental functioning in adulthood [Danese et al., 2007; McCutcheon et al., 2009; Shonkoff et al., 2009], possibly by altering the neural networks that underpin some affective–cognitive processes [Goodyer, 2002]. Therefore, we also analyzed the differential impact of LE at different epochs of life, namely childhood–adolescence as opposed to adulthood.

Outcome Variables

Post 35% CO_2/65% O_2 challenge VASA scores yield skewed distributions, without an a priori ideal threshold to define a positive response [Battaglia and Perna, 1995; Rassovsky and Kushner, 2003; Battaglia et al., 2007] to the challenge. Therefore, as in our previous

studies [Battaglia et al., 2007, 2008b, 2009], we adopted two thresholds—the 75th and 90th Δ% VASA score percentiles of the sample—whereby each participant could be classified as a responder or non-responder. The 90th percentile corresponds here to the 26% increment of anxiety, which best discriminates people with PD from controls according to ROC analysis [Battaglia and Perna, 1995]. The 75th percentile was set as a more lenient threshold, suitable for participants from the general population [Battaglia et al., 2007]. Likewise, since categorically defined PD is relatively rare in the population [Kendler et al., 1993; Kendler et al., 2001], as in previous population studies [Battaglia et al., 2008b, 2009], we organized the CIDI diagnostic information into three categories: 0, unaffected (participant had never experienced a spontaneous panic attack); 1, broad PD (participant had experienced $\geq$1 panic attacks, but failed to meet the full lifetime/current diagnosis) and 2, narrow PD (participant satisfied lifetime/current criteria for *DSM-IV* PD). Previous controls for the appropriateness of this approach via multiple threshold tests for MZ and DZ twins for each of these two phenotypes provided good fits [Battaglia et al., 2009], suggesting that, within the limits of these data and procedures, the milder and stronger responses to CO_2 and broad and narrow PD are on the same continua of liability [Kendler et al., 1993, 2001; Battaglia et al., 2007, 2008b].

We have already shown that both the "major life events" and the "stressful life events" variables predict significantly the response to the 35% CO_2/65% O_2 challenge and the lifetime diagnosis of DSM-IV PD, and that major LE and stressful LE were marginally correlated [Ogliari et al., 2010] (Pearson's r between −0.09 and 0.07) in this sample [Ogliari et al., 2010].

Analytic Approach

For all genetic analyses, models were fitted to raw data using the Mx software [Neale et al., 2003] for maximum likelihood analyses. First, we analyzed the causal structure of the NUM and SEV moderators by univariate analyses that decompose the phenotypic variance into additive genetic influences (A), environmental effects that are shared by twins (C), and unique environmental influences (E). Such decomposition is possible by comparing phenotypic resemblance in MZ and DZ twins while assuming a correlation between twins' additive genetic influences of 1.0 for MZ pairs and of 0.5 for DZ pairs, and a correlation between twins' shared environmental influences of 1.0 for both MZ and DZ twin pairs (i.e., shared environmental influences are assumed of equal magnitude for MZ and DZ twins).

Then, we tested the hypothesis that genetic and environmental effects upon the liability to: (a) CO_2 sensitivity (as indexed by post-35% CO_2/65% O_2 anxiety measured with the Δ% VASA) and (b) DSM-IV PD (as indexed by the CIDI interview) vary across different levels of the environmental moderators NUM and SEV. To do so, we implemented a G×E interaction model for ordinal data. This model [Medland et al., 2009] extends within the context of the liability threshold model and ordinal phenotypes, the model originally developed by Purcell [2002] for G×E analyses of continuously distributed variables. To test for the environmental moderation of genetic effects on phenotypes, the model [Medland et al., 2009] incorporates interaction coefficients into univariate models. Figure 1 shows that the genetic path to the phenotype is redefined to include a moderation term which represents a linear function of the environmental moderator [Purcell, 2002]. Compared to the simpler linear-additive model, in the G×E approach, the additive genetic path is no longer simply "a", but it becomes $a + \beta_x M$, where "a" is an estimate of the main genetic effect, and β_x represents a regression coefficient indicating to what extent genetic effects are a linear function of the environmental moderator (M). If β_x is significantly different from zero, then an A×M interaction is present. In a similar fashion, the model allows for testing the presence of shared environmental and unique environmental interaction effects (respectively: C×M and E×M), which are assessed by the significance of the respective β_y and β_z regression coefficients. Moreover, the model includes a main—or mediating—effect of the moderator upon the phenotype's mean/thresholds [Purcell, 2002] which is mapped by the β_M parameter, even though the decomposition of variance is not influenced by this effect.

When working with ordinal data, in order to ensure identification of the model parameters, the latent liability distribution must be identified upon an arbitrary scale. This restriction is usually imposed by constraining the estimated variance of the ordinal variable to unity [Medland et al., 2009], thus yielding standardized estimates of variance components. However, this approach does not allow for the estimation of the absolute magnitude of the genetic and environmental variance, a relevant piece of information in the context of G×E analysis. A G×E effect is implied by a significant variation in the absolute—but not necessarily in the standardized—genetic variance, as a function of an environmental moderator. As a solution to this problem when dealing with polichotomous data, the model developed by Medland et al. [2009] fixes any two thresholds of the ordinal phenotype, instead

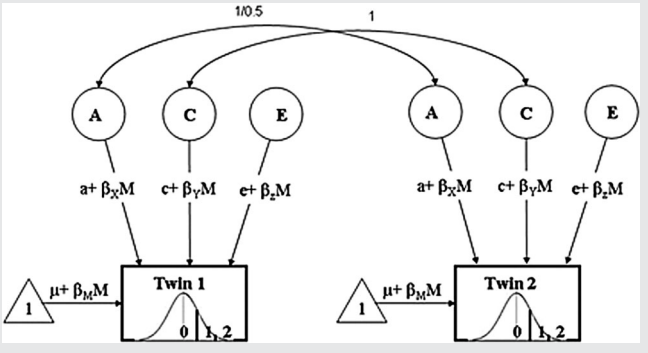

FIG. 1. Univariate model of gene–environment interaction for polichotomous data in the context of the liability threshold model. A, additive genetic effects; C shared environmental effects; E, non-shared environmental effects; M, environmental moderator; *a*, un-moderated proportion of additive genetic variance; β_x, moderated proportion of additive genetic variance; *c*, un-moderated proportion of shared environmental variance; β_y, moderated proportion of shared environmental variance; *e*, un-moderated proportion of non-shared environmental variance; β_z, moderated proportion of non-shared environmental variance; μ, un-moderated proportion of mean; β_M, effect of the moderator upon the phenotype's mean/thresholds.

of constraining the variance. This restriction is sufficient to identify the latent liability distribution upon an arbitrary scale [Mehta et al., 2004]. Therefore, we fixed to 0 and 1 the two thresholds for both the VASA and the PD phenotypes. We fitted all G×E models with A and E only, on the basis of our previous quantitative genetic analysis for both post-35% CO_2/65% O_2 VASA and DSM-IV PD in this same sample [Battaglia et al., 2008b]. In seeking to prevent some of the computational problems that may arise by the correlation between the main- and the interaction effects, we z-standardized the NUM and SEV moderators before the model fitting analyses [Aiken and West, 1991].

The models' fit were evaluated using hierarchical χ^2 tests, as the difference between twice the negative log likelihood (−2LL) for the reduced and the full models have a χ^2 distribution, with df given by the difference between the df for the two models [Heath et al., 1989]. Models were also compared on the basis of the Akaike information criterion (AIC = −2LL-2 df), with the lowest AIC value reflecting a balance between goodness of fit and parsimony.

RESULTS

An examination of the distributional properties of untransformed NUM and SEV (NUM: Range 0–14; mean ± SD: 5.39 ± 2.10; skewness: 0.58; SEV: Range 0–29, mean ± SD: 9.21 ± 5.06, skewness 0.99) showed a monotonic increase for both moderators as a simple time function. Preliminary analyses were carried out to: (a) evaluate the ability of NUM and SEV to predict PD/CO_2 response and (b) confirm the degree of independence of NUM and SEV from other predictors of PD/CO_2 response. Regression analyses in twins considered as individuals carried out in STATA [STATA, 2005] with the "robust" option to compensate for the lack of independence of observations [Carlin et al., 2005], showed that z-transformed NUM and SEV predicted both lifetime DSM-IV PD and post-CO_2 VASA with significance values that ranged from $P = 0.02$ (NUM on post-CO_2 VASA) to $P = 0.0001$ (SEV on post-CO_2 VASA), paralleling our previous findings that major LE and stressful LE—considered separately and defined by different metrics and measures [Ogliari et al., 2010]—predict PD and the CO_2 response in this sample.

Both NUM and SEV correlated only modestly with other variables—including CPL, events of suffocative nature, symptoms of SAD—that we had previously found [Ogliari et al., 2010] to predict PD and the CO_2 response. The smallest correlation (Pearson $r = 0.02$) was observed between NUM and SAD symptoms; the largest (point-biserial $r = 0.16$) was observed between SEV and CPL, confirming the substantial independence of LE from other predictors of PD and CO_2 sensitivity included in our research plan [Ogliari et al., 2010].

Table II shows the variance components and the fit indices of the univariate models for the NUM and SEV moderators. In the full models the heritability estimates were 0.15 (CI: 0.0–0.44) and 0.07 (CI: 0.0–0.43), respectively: These figures are in agreement with those of a review of 55 independent studies [Kendler and Baker, 2007], which yielded weighted heritability estimates between 7% and 39% (with most estimates falling between 15% and 35%) for measures of the environment and stressful LE.

In both best-fitting models the additive genetic component could be dropped without significant worsening of fit, and an entirely environmental (both shared and unique) causal structure could be assumed for both moderators.

Table III reports the fit statistics for the models of gene–environment interaction. Both the NUM and SEV moderators exerted moderation effects on the genetic and unique environmental variance of post-CO_2 VASA scores, as indicated by the significant fit deterioration when either moderation term β_x or β_z were dropped from the models. On the other hand, neither genetic nor unique environmental components of DSM-IV PD appeared to be moderated by NUM or SEV, as shown by the non-significant fit deterioration when dropping the moderated components.

Figure 2 provides a graphical representation of the moderation effects on post-CO_2 VASA (part A) and DSM-IV PD (part B), by depicting the absolute values of genetic and environmental variance across different levels of the environmental risk factors expressed in SD units.

Both the additive genetic and the non-shared environmental variance of post-CO_2 VASA increased with greater NUM and SEV values, while the variance components of DSM-IV PD remained stable across different levels of the moderators. However, we note

TABLE II. Univariate Variance Components Estimates and Model Fitting Indices for Cumulative NUM and SEV of LE

		Variance components			Model fitting indices					
Moderator	**Model**	**A**	**C**	**E**	**−2LL**	**df**	**Akaike**	**Change in χ^{2a}**	**df**	***P* value**
Number	ACE	0.153	0.166	0.681	1879.323	681	517.323			
	AE	0.351	—	0.649	1880.524	682	516.524	1.201	1	0.273
	CE[b]	—	0.276	0.724	1879.949	682	515.949	0.626	1	0.429
	E	—	—	1	1907.194	683	541.194	27.871	2	0.000
Severity	ACE	0.071	0.260	0.669	1893.607	683	527.607			
	AE	0.379	—	0.621	1896.660	684	528.660	3.053	1	0.081
	CE[b]	—	0.311	0.689	1893.749	684	525.749	0.142	1	0.706
	E	0	0	1	1928.547	685	558.547	34.941	2	0.000

A, additive genetic effects; C, shared environmental effects; E, non-shared environmental effects; −2LL, twice the negative log likelihood.[a]The change in χ^2 is the difference between the −2LL in the full model and in the model tested.
[b]Best-fitting model.

TABLE III. Comparisons for Gene–Environment Interaction Models on CO_2 Sensitivity and PD With Cumulative NUM and SEV of LE as Moderators

Phenotype	Moderator	Model[a]	−2LL	df	Akaike	Change in χ^{2b}	df	*P* value
CO_2 sensitivity	Number	1. Full[c]	951.330	673	−394.670	—	—	—
		2. $\beta_x = 0$	958.293	674	−389.707	6.963	1	0.008
		3. $\beta_z = 0$	955.239	674	−392.761	3.909	1	0.048
	Severity	1. Full[c]	942.081	673	−403.919	—	—	—
		2. $\beta_x = 0$	948.396	674	−399.604	6.315	1	0.012
		3. $\beta_z = 0$	946.394	674	−401.606	4.313	1	0.038
Panic Disorder	Number	1. Full	356.101	653	−949.899	—	—	—
		2. $\beta_x = 0$	356.355	654	−951.645	0.254	1	0.614
		3. $\beta_x = 0$ and $\beta_z = 0$[c]	357.260	655	−952.740	1.159	2	0.560
	Severity	1. Full	345.091	653	−960.909	—	—	—
		2. $\beta_x = 0$	346.711	654	−961.289	1.620	1	0.203
		3. $\beta_x = 0$ and $\beta_z = 0$[c]	347.067	655	−962.933	1.976	2	0.372

β_x, moderated proportion of additive genetic variance; β_z, moderated proportion of non-shared environmental variance; −2LL, twice the negative log likelihood.[a]"Full" refers to a gene–environment interaction model encompassing both the genetic and the non-shared environmental moderation parameters.
[b]The change in χ^2 is the difference between the −2LL in the full model of reference and in the model tested.
[c]Best-fitting model.

that these estimates are generated via mathematical models that impose linear changes in the genetic and environmental variances across different levels of the moderators, and do not necessarily depict point-by-point measured effects arising from experimental manipulation.

Table IV shows the unstandardized estimates of variance from the best-fitting models for post-CO_2 VASA and DSM-IV PD. The higher β_x value for SEV than for NUM implies higher genetic variance under the SEV, than the NUM moderation model. This means that the moderation effect of LE upon genetic variance for

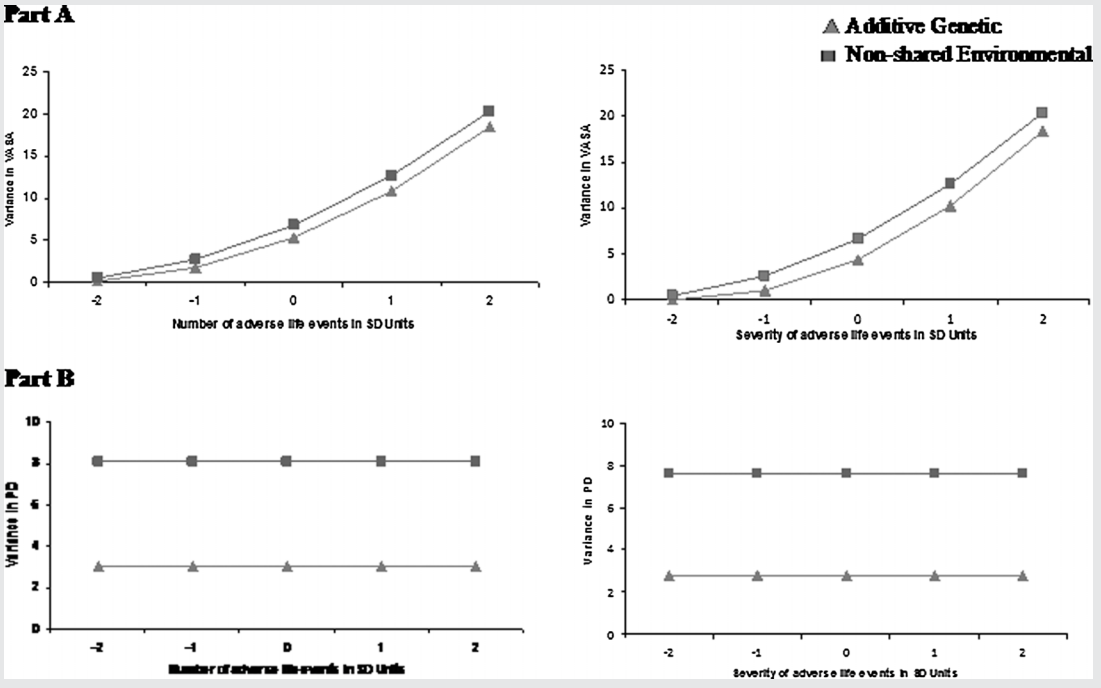

FIG. 2. Moderation effects on CO_2 sensitivity (part A) and PD (part B) of the cumulative NUM and SEV of LE. The x-axis provides the level of the environmental moderator in SD units: higher values indicate greater environmental adversity. The y-axis provides the level of the unstandardized additive genetic (A) and environmental (E) variance for CO_2 sensitivity (part A), and PD (part B). A NUM z-score of −2 in the graph corresponds to one LE, while a NUM z-score of +2 is equivalent to six events. A SEV z-score of −2 corresponds to a perceived SEV of 0, while a SEV z-score of +2 is equivalent to a cumulative SEV score of 17.

TABLE IV. Unstandardized Variance Components Estimates From Best Fitting Models Shown in Table II

Phenotype	Moderator	a	β_x	e	β_z
CO_2 sensitivity	Number	2.3039	0.9963	2.5935	0.9595
	Severity	2.1058	1.0912	2.5738	0.9718
Panic disorder	Number	1.7393	—	2.8434	—
	Severity	−1.6629	—	2.7627	—

a, un-moderated proportion of additive genetic variance; β_x, moderated proportion of additive genetic variance; e, un-moderated proportion of non-shared environmental variance; β_z, moderated proportion of non-shared environmental variance.

CO_2 response becomes more evident when the SEV variable is employed. For instance, at a value of 2 SD for the LE, the β_xM values would be 2.18 and 1.98, respectively, for SEV and NUM. Moreover, the results of the ACE full models (see Table II) showed that SEV had a smaller genetic component than the NUM moderator (7% vs. 15%), which would minimize the probability of gene–environment correlation with the phenotype under study (the post-CO_2 response in our case). In the light of these considerations, we investigated the hypothesis that the moderation of LE upon genetic variance for CO_2 reactivity varies across different lifetime windows of risk via the SEV variable.

Consistently with previous genetic epidemiological investigations of LE/adversities in PD and internalizing disorders among general population twin subjects [Kendler et al., 1992; McCutcheon et al., 2009] and in this same sample [Battaglia et al., 2009; Ogliari et al., 2010] we specified a childhood–adolescence window of risk for LE having occurred before the 18th birthday, and an adulthood window of risk between the 18th birthday and age-at-interview. According to this dichotomy, we obtained for every subject two separate measures of cumulative SEV of LE: SEVc–a (covering the cumulative SEV of LE before the 18th birthday), and SEVadult (covering the cumulative SEV of LE endorsed from age 18 to the day of the interview). The correlation between SEVadult and SEVc–a was very modest ($r = 0.058$, $P = 0.12$), and twins reported higher SEVadult than SEVc–a values ($7.39 + 4.21$ vs. $1.89 + 2.73$, $P = 0.001$). This finding could reflect a higher probability of facing LE in adulthood, and/or a regency effect [Rubin, 1999; Janssen et al., 2005].

Regressions in twins considered as individuals, however, showed that SEVc–a was a better predictor of CO_2 responses than SEVadult (regression of z-transformed SEVc–a on post-CO_2 VASA: Beta = 0.15, $P = 0.001$; regression of z-transformed SEVadult on post-CO_2 VASA: Beta = 0.11, $P = 0.003$).

Table V shows that when we modeled the SEV effect separately for SEVc–a and SEVadult, only the LE that had occurred during childhood–adolescence yielded results compatible with G×E effects. For SEVc–a the best-fitting model (model 3) allowed for dropping the E-moderated effect (thus excluding an E-by-E interaction), but had a significant worsening of the fit if the A-moderated effect was dropped. On the contrary, for SEVadult the best fitting model allowed for dropping all (A and E) moderated components of variance.

The unstandardized estimates of genetic and environmental variance influencing post-CO_2 VASA from the best-fitting model using SEVc–a as a moderator were: 1.6977 (a) and 0.6995 (β_x) and 1.9162 (e), respectively.

DISCUSSION

Our data show that interactional effects should be taken into account over and above the additive effects to explain individual differences for reactivity to CO_2, a putative endophenotype of PD. Accordingly, the genetic susceptibility to CO_2 response may influence the sensitivity to environmental hazard, or LE may enhance the effect of genetic influences on CO_2 sensitivity. Moreover, results suggest that LE occurring early in life can alter chemoception via G×E mechanisms, with effects that can persist beyond the developmental years. Inasmuch as the trait of heightened sensitivity to CO_2 mapped by the 35% CO_2/65% O_2 challenge is a valid neurofunctional component of PD, our findings can help revealing one of

TABLE V. Comparisons for Gene–Environment Interaction Models of CO_2 Sensitivity and Environmental Moderators

Moderator	Model[a]	−2LL	df	Akaike	Change in χ^{2b}	df	*P* value
Severity of LE in childhood–adolescence	1. Full	850.580	652	−453.420	—	—	—
	2. $\beta_x = 0$	855.092	653	−450.908	4.512	1	0.034
	3. $\beta_z = 0$[c]	851.439	653	−454.561	0.859	1	0.354
Severity of LE in adulthood	1. Full	954.092	671	−387.908	—	—	—
	2. $\beta_x = 0$	956.031	672	−387.969	1.939	1	0.164
	3. $\beta_x = 0$ and $\beta_z = 0$[c]	958.042	673	−387.958	3.950	2	0.139

β_x, moderated proportion of additive genetic variance; β_z, moderated proportion of non-shared environmental variance; −2LL, twice the negative log likelihood.[a]"Full" refers to a gene–environment interaction model encompassing the genetic and non-shared environmental moderation parameters.
[b]The change in χ^2 is the difference between the −2LL in the full model and the others models tested.
[c]Best fitting model.

the pathogenetic routes by which early life experiences bring about continuities and discontinuities in PD and its related psychobiology.

For the corresponding phenotype of DSM-IV PD, however, results showed the explanatory sufficiency of the simpler, additive variance component model. Similarly negative, or ambiguous, results regarding G×E effects are commonly seen in quantitative genetic studies of human behavioral disorders [Eaves, 2006; Rutter et al., 2006; Lau et al., 2007]. Since, however, the response to the 35% CO_2/65% O_2 challenge and DSM-IV PD share a conspicuous amount of additive genetic variance [Battaglia et al., 2008b], the possible reasons for this inconsistency of results need be discussed. If the main reason is simply that of power—whereby the number of subjects with lifetime PD was about half that of "responders" to the CO_2 challenge [Battaglia et al., 2008b], then future studies in larger twin samples will find G×E effects on DSM-IV PD. If the reason resides mainly in the ability of the endophenotype to map some functional pathways lying between the latent liability and the behavioral disorder, and these pathways are sensitive to G×E mechanisms, then even future studies in larger twin samples will confirm our results, i.e., yield evidence for G×E on CO_2 sensitivity, but not necessarily on DSM-IV PD. Moreover, as noted by Kendler and Neale [2010], a model that assumes that all genetic effects that have an impact on an endophenotype also affect the corresponding phenotype, is unrealistic. It appears biologically more realistic that amongst all genetic effects, some influence both CO_2 sensitivity and PD, whereas some will be exclusive of the former, or of the latter. Consequently, it is conceivable that we have detected G×E effects that are proper of the pathophysiological pathways of CO_2 sensitivity, and not necessarily of PD.

Six potential limitations apply to this study. First, we indexed CO_2 sensitivity by the anxious response to a CO_2 challenge, which could be partially mediated by unmeasured neuroticism/anxiety sensitivity [Coryell et al., 2006; Schmidt and Zvolensky, 2007]. However, very recent data [Ziemann et al., 2009] show that the amygdala is itself a chemosensor that initiates fear responses under hypercarbia and acidosis. Thus, the anxious response to the 35% CO_2/65% O_2 challenge can at least partially constitute a direct readout of hypercarbic stimulation on the brain's limbic circuitries. Second, the participants' ability in recalling, and reliability in rating LE necessarily influenced the NUM and SEV indexes. Interviewer-based, rather than subjective, measures of LE severity [Brown and Harris, 1989; Kendler et al., 1998; Kendler and Prescott, 2006] are considered more reliable. However, a major source of bias in this domain would be that of overemphasizing the association between LE and overt psychopathology. Inasmuch as NUM and SEV moderate the genetic variance for the response to a biological challenge—not a disorder—a "seeking after meaning" bias [Kendler and Prescott, 2006] is unlikely to have affected the G×E results with the CO_2 response. Moreover, objective assessment of LE severity often implies the probing of participants upon LE-related themes [Kendler and Prescott, 2006] including maltreatment, which may in turn elicit variable degrees of emotionality. Here, we needed to limit the stress connected to the LE assessment because the interview was followed by the CO_2 challenge, which is itself stressful.

Third, while NUM and SEV are different indices, they are obviously related, and were—as expected—highly correlated ($r = 0.85$) in this sample. It could then be argued that separate analyses of their moderating effects are redundant. To estimate the impact of the overlap between the two moderators, we repeated the G×E analyses on post-CO_2 VASA by deriving a moderator from the residual of the correlation between SEV and NUM (i.e., after partialling the effect of the number of LE out of the severity of LE). Interestingly, in spite of the loss of power implied by such maneuver, a comparison of a model that excluded the A-moderated effect to the full (moderated) AE model, yielded a worsening of fit with a strong trend toward significance (−2LL: 954.953, df = 674, $\chi^2_1 = 2.934$; $P = 0.08$; AIC = −393.047), while a model that excluded the E-moderated term had no significant worsening of fit (−2LL: 952.100, df = 674, $\chi^2_1 = 0.082$; $P = 0.78$; AIC = −395.900).

Fourth, the model of G×E effects used here does not allow to differentiate gene–environment interaction from gene–environ-environment correlation. Therefore, part of the estimated G×E effect could encompass an undetected proportion of G×G effects, due to an overlap between the genes that influence variation in LE and genes that influence variation in post-CO_2 anxiety. However, the univariate analyses of our LE moderators (Table II) showed, even for the full models, small genetic components, so that extensive confounding caused by untreated gene–environment correlations appears unlikely. Fifth, our indexes—NUM and SEV—of LE, and our computational approaches preclude a clear separation of "age at first exposure" effects from "duration of exposure" effects [Costello and Angold, 1995]. Likewise, the cross-sectional nature of the study does not allow to differentiate stable- from age-specific genetic effects, nor to state exactly when G×E becomes effectual during development [Lau et al., 2007]. Sixth, this sample is relatively small for G×E analyses even thought this is probably the largest twin sample ever probed for CO_2 reactivity; reduced samples remain however inevitable constraints of studies that use stressful procedures.

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ORIGINAL ARTICLE

A Genetically Informed Study of the Association Between Childhood Separation Anxiety, Sensitivity to CO_2, Panic Disorder, and the Effect of Childhood Parental Loss

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Context: Childhood separation anxiety disorder can predate panic disorder, which usually begins in early adulthood. Both disorders are associated with heightened sensitivity to inhaled CO_2 and can be influenced by childhood parental loss.

Objectives: To find the sources of covariation between childhood separation anxiety disorder, hypersensitivity to CO_2, and panic disorder in adulthood and to measure the effect of childhood parental loss on such covariation.

Design: Multivariate twin study.

Participants: Seven hundred twelve young adults from the Norwegian Institute of Public Health Twin Panel, a general population cohort.

Main Outcome Measures: Personal direct assessment of lifetime panic disorder through structured psychiatric interviews, history of childhood parental loss, and separation anxiety disorder symptoms. Subjective anxiety response to a 35% CO_2/65% O_2 inhaled mixture compared with compressed air (placebo).

Results: Our best-fitting solution yielded a common pathway model, implying that covariation between separation anxiety in childhood, hypersensitivity to CO_2, and panic disorder in adulthood can be explained by a single latent intervening variable influencing all phenotypes. The latent variable governing the 3 phenotypes' covariation was in turn largely (89%) influenced by genetic factors and childhood parental loss (treated as an identified element of risk acting at a family-wide level), which accounted for the remaining 11% of covariance. Residual variance was explained by 1 specific genetic variance component for separation anxiety disorder and variable-specific unique environmental variance components.

Conclusions: Shared genetic determinants appear to be the major underlying cause of the developmental continuity of childhood separation anxiety disorder into adult panic disorder and the association of both disorders with heightened sensitivity to CO_2. Inasmuch as childhood parental loss is a truly environmental risk factor, it can account for a significant additional proportion of the covariation of these 3 developmentally related phenotypes.

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THE TYPICAL ONSET OF PANIC disorder (PD) occurs in early adulthood,[1] but a closer investigation of one's developmental years may increase our understanding of this illness. Relatively distinct behavioral and psychophysiological antecedents have been described for PD.[2,3] Some adverse events occurring early in life appear to increase the risk of later manifesting the disorder in addition to—or in interaction with—familial genetic causal factors.[4,5]

An operationally defined abnormal childhood behavior that may represent an antecedent to adult-onset PD is separation anxiety disorder (SAD).[6,7] Separation anxiety disorder has been found to be specifically associated with heightened individual risk to develop PD in controlled, long-term follow-up studies of clinical and nonclinical pediatric samples[8,9] and in retrospective studies of adults.[10] Yet, some authors have not found an association between SAD and panic attacks.[11] Moreover, epidemiological data alone are not powerful enough to clarify the nature of continuity[12] between SAD and PD, which could reflect ongoing genetic or environmental influences or a combination of the two.

Several studies of CO_2 sensitivity in children and adults offer an opportunity to assess a neural substrate common to SAD and PD. Controlled studies of children and adolescents with anxiety show a consistent association of SAD with CO_2 hypersensitivity, both defined by symptom reports,[13,14] and with abnormal respiratory measures during exposure to hypercap-

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nia.[14] The responses to CO_2 stimulation in children with SAD thus appear similar to those seen in adults with PD.[13,15,16]

At least 3 potential limitations apply, however. First, although longitudinal designs would best address the question of the extent to which childhood SAD predicts CO_2 hypersensitivity in adulthood, our study uses retrospective assessments of SAD symptoms in adults who underwent CO_2 stimulation. Second, the possible role of other diagnoses, anxiety disorders most prominently, in influencing the subjective anxiety response to CO_2 stimulation should be considered, given the rates of comorbid disorders described for both PD[1] and SAD.[14] Third and foremost, the underlying causes of covariation between reported symptoms of SAD in the developmental years, CO_2 reactivity, and PD in adulthood remain unaddressed.

Turning to the role of early life experiences in bringing about continuities and discontinuities in psychopathology, actual separation events during childhood (encompassing, eg, parental death, separation or divorce, relocation, sometimes cumulatively referred to as childhood parental loss [CPL][4]) have been recognized as risk factors to predict PD in adulthood by general population and clinical studies.[4,17] On the other hand, studies of adults with PD[18] that found that both SAD and separation events were associated with PD reported a near-zero correlation between actual separation experiences and childhood SAD. This may mean that at least partially independent developmental pathways lead from parental loss and/or excessive worry about separation in childhood to PD in adulthood. However, heterogeneity may act as a confounder: if both PD and SAD were heterogeneous, failure to distinguish between subgroups would reduce the correlations.

The present study sought to address some of these issues within a genetically informed design. In a sample of young adult twins from the Norwegian Institute of Public Health Twin Panel, we examined 3 principal questions. First, we wanted to clarify the sources of covariation between retrospectively assessed SAD symptoms, sensitivity to CO_2, and the emergence of PD in adulthood. Second, we wanted to understand whether the genetic and/or environmental causes of such covariation are best conceived as acting directly and independently on phenotypes or through the mediation of an additional common higher-order factor. Third, we wanted to assess whether, and to what extent, adding CPL as an identified element of risk to a causal model of covariation between SAD, sensitivity to CO_2, and PD would improve our ability to explain the nature of these associations.

METHODS

RECRUITMENT AND ASSESSMENT

As outlined in detail elsewhere (A.O. et al, unpublished data, 2008),[19,20] from 2002 to 2004 we consecutively recruited a sample of 712 subjects (346 complete pairs plus 20 single twins) from the Norwegian Twin Study on the Genetics of Personality and Mental Health (a cohort sequential design program based on a general population cohort of twins). From these twins, we gathered extensive information on CO_2 reactivity, occurrence of life events, antecedents of PD, and direct psychiatric evaluations. Although the large majority of participants were randomly ascertained to guarantee a sufficient number of individuals who would be informative at the CO_2 challenge, 12% of pairs were selectively ascertained on the endorsement of anxiety-related items in a mailed questionnaire they had answered in 1998.[19] Despite the partially nonrandom ascertainment, factors such as age, frequency of contact with co-twin, and self-rated symptoms of generalized anxiety disorder or depression had no or marginal ability to predict participation in the CO_2 study.[19] To assess individual sensitivity to CO_2, we used the 35% CO_2 single-breath test,[19,21] which is a safe procedure with a good ability to distinguish patients with PD from controls and patients with other mental disorders.[16,22,23]

After complete description and screening of exclusion criteria,[19,21] signed informed consent was obtained as approved by the Regional Committee for Research Ethics and the Norwegian government. Two gas mixtures were used: compressed air (placebo) and a mixture of 35% CO_2 and 65% O_2. Participants inhaled the gasses through a self-administration mask connected to a Mark 20 Wright respirometer (Ferraris Medical, Hertford, England) to measure vital capacity and the gas volume delivered at each inhalation. Participants were informed before the challenge that they would inhale 2 harmless gas mixtures that might elicit some discomfort, from few physical symptoms to clear anxiety. After vital capacity was measured, the participants inhaled 1 vital capacity of compressed air. Then, after an interval of 30 minutes, they inhaled 35% CO_2/65% O_2. At the end of each inhalation, participants held their breath for 4 seconds. According to this standardized procedure,[21,23] the test is valid if at least 80% of vital capacity is inhaled.

In the interval between the 2 inhalations, 2 interviewers (trained and supervised by M.B. and K.T.) who were masked to participants' diagnoses investigated, using a semistructured interview, the participants' lifetime history of stressful events, SAD, and CPL (A.O. et al, unpublished data, 2008). The occurrence of symptoms of SAD was retrospectively assessed by the 12-item questionnaire by van der Molen et al[24] adapted for *DSM-IV* criteria.[7] As in previous studies of adults,[4] CPL was defined as a period of at least 1 year of unexpected or unscheduled separation from 1 or both biological parents that occurred prior to the participants' 17th birthday. As part of the Norwegian Twin Study on the Genetics of Personality and Mental Health,[25] all twins were also interviewed for lifetime Axis I and II disorders with the Composite International Diagnostic Interview (CIDI)[26] and the Structured Clinical Interview for *DSM-IV* Personality,[27] which were administered by experienced psychology students or psychiatric nurses who had adequate training.[28]

OUTCOME VARIABLES

The response evoked by the 35% CO_2/65% O_2 test was measured by the Panic Symptom List III–Revised[29] and the Visual Analog Scale for Anxiety (VASA).[30] Because post-CO_2 VASA scores have better reliability[31] and discriminative power,[22] we based our study on these scores (0, no anxiety at all; 100, the worst anxiety imaginable) obtained immediately after inhalation.

After the 35% CO_2/65% O_2 test, self-rated anxiety scores typically yield skewed distributions without an a priori ideal threshold to define a positive response.[16,19,22] To deal with these issues, we adopted 2 thresholds—the 75th and 90th VASA score percentiles of the sample—whereby each participant could be classified as a responder or nonresponder. The 90th percentile corresponds here to the 26% increment of anxiety, which has been shown[22] to be the ideal threshold to distinguish people

with PD from controls, while the 75th percentile threshold was set to identify a more lenient level of sensitivity to hypercapnia, suitable for participants from the general population.[19]

Likewise, because categorically defined (present or absent) PD is relatively rare in the population,[1,32,33] we organized CIDI diagnostic information into 3 categories: 0, unaffected (participant had never experienced a spontaneous panic attack); 1, broad PD (participant had experienced ≥1 panic attacks, but failed to meet the full lifetime diagnosis or to have a current diagnosis of *DSM-IV* PD); and 2, narrow PD (participant satisfied lifetime or current criteria for *DSM-IV* PD). As we did with VASA scores and PD, we imposed 2 thresholds (the 75th and 90th percentiles of the sample) on the semicontinuously distributed SAD scores, whereby each participant could be classified as 0, unaffected; 1, having mild separation anxiety; or 2, having substantial separation anxiety/SAD. Owing to limited power, CPL was sorted dichotomously (0, no CPL; 1, CPL) without differentiating between parental death, divorce, continued separation, or maternal vs paternal CPL.

By specifying 2 thresholds on all traits, we adopted a multiple threshold model[34] approach, which assumes different degrees of severity on the same normally distributed underlying continuum of risk. Controls for the appropriateness of this assumption made by multiple threshold tests with PRELIS[35] for monozygotic (MZ) and dizygotic (DZ) twins for each of the 3 phenotypes provided good fits (range, MZ twin relative to post-CO_2 VASA score, $P=.12$; MZ twin relative to SAD, $P=.89$), suggesting that, within the limits of these data and procedures, the milder and stronger responses to CO_2 and broad and narrow SAD and PD are on the same continua of liability, as has been consistently shown in previous reports.[19,20,32,33]

CLINICAL AND SOCIODEMOGRAPHIC CHARACTERISTICS OF THE SAMPLE

The characteristics of the sample have been detailed elsewhere (A.O. et al, unpublished data, 2008).[19,20] Briefly, of 712 participants, 64% were women, 49% were MZ twins, and the mean age was 30.95 years (standard deviation, 3.6 years). No participant had an anxious response to inhalation of compressed air (placebo). A lifetime diagnosis of *DSM-IV* PD was present in 6.6% of participants, and a retrospective diagnosis of SAD was attributed to 10.4% of participants for having endorsed 3 or more SAD symptoms for at least 1 month and having significant interference during childhood to adolescence. Both values are consistent with those reported in people in the general population of comparable age who were interviewed for lifetime PD[36] and retrospectively for SAD.[37]

Causes of CPL included parental death (4.1% of participants) and separation events (divorce, job relocation, military service, etc, endorsed by 11.1% of participants). These were reported concordantly by the large majority of twin pairs. The few cases in which only 1 twin in a pair had reported CPL were, after inspection, attributable to minor discrepancies (usually a few months) in dating the occurrence of a separation event shortly before or after the twins' 17th birthday. Therefore, to increase sensitivity, all pairs in which at least 1 twin had reported CPL were considered concordant-positive (17.7%), while all other pairs were concordant for not having experienced CPL (82.3%). Consequently, herein CPL is considered a fully concordant family-wide environmental element of risk.

STATISTICAL ANALYSIS

Preliminary analyses with logistic regression (model $\chi^2_2=30.621$, $P<.001$) showed that positive responses to CO_2 were predicted by SAD (Wald $\chi^2=22.1$; odds ratio, 2.75; 95% confidence interval, 1.8-4.2; $P<.001$) and PD (Wald $\chi^2=4.3$; odds ratio, 2.09; 95% confidence interval, 1.1-4.2; $P=.04$) but not by lifetime diagnoses of social phobia, obsessive-compulsive disorder, generalized anxiety disorder, depression, or blood or injury phobias on the CIDI. Amongst the nonsignificant predictors mentioned, depression had the largest effect ($P=.26$). Likewise, when the correlations between post-CO_2 VASA score and PD and between post-CO_2 VASA score and SAD were controlled for each of the aforementioned lifetime diagnoses on the CIDI, we observed only modest variations of correlation coefficients (mean variation in correlation coefficients, 6.5%; minimum variation, 0.1% between PD and VASA score after controlling for generalized anxiety disorder; maximum variation, 15% decrease of correlation between PD and VASA score after controlling for depression), all of which remained significant. Therefore, our multivariate analyses encompassed PD and SAD but no other lifetime diagnoses on the CIDI.

All structural equation modeling analyses were run with the Mx program[38] using raw data, including the twin pairs with incomplete information by using the method of maximum likelihood. The within-twin and cross-trait (between 2 traits in the same twin), the cross-twin and within-trait (between twin 1 and twin 2 of the same pair for the same trait), and the cross-twin and cross-trait (between 1 trait in 1 twin and the other trait in the co-twin) polychoric correlations (based on the assumption of an underlying continuous bivariate normal liability distribution) were obtained by running the script *ordSATmt2.mx,* available in the Mx library.[39] Owing to limited power, we did not explore possible sex-specific variance effects. The polychoric correlations were then calculated by assuming no sex differences and by only using 2 zygosity categories: MZ and DZ. We began estimations of the polychoric correlations by calculating the relative fit of a model without constraints (ie, a saturated model, which contains as many parameters as there are unknowns), against which we compared the fit of simpler models with progressively more elaborated constraints. We successively tested the likelihood of imposing no difference in thresholds between the first and second twins in a pair and MZ and DZ pairs for SAD, positive post-CO_2 response measured with the VASA, and PD (all 3 traits under categories 0, 1, 2, as explained in the "Outcome Variables" section).

We then applied a multivariate twin design to the data. A multivariate twin design models the causal sources of covariation between 3 or more conditions and allows for the separation of the total phenotypic variance and covariance of traits into proportions owing to (1) additive genetic, (2) shared environmental (eg, experiences associated with socioeconomic and/or religious background), and (3) unique (individual-specific) environmental (like most illness, interpersonal relationships, etc) factors. The models compare MZ and DZ twins' phenotypic resemblance, assuming correlations of 1.0 for MZ pairs and 0.5 for DZ pairs between their additive genetic influences (DZ twins share half of their segregating genes on average) and a correlation of 1.0 for both MZ and DZ pairs between their shared environmental influences; unique environmental influences are uncorrelated for all twin pairs (with the equal environment assumption). While the purpose of the univariate twin design is to explain the causes of individual differences for a single phenotype, multivariate models involve twin correlations for different traits taken into account simultaneously and thus can be viewed as a simultaneous factor analysis on the genetic and environmental variances and covariances.[40] By comparing the cross-trait twin correlations in MZ and DZ twins, the sources of covariance between the traits are quantified so that greater MZ than DZ cross-twin cross-trait correlations suggest genetic influences on covariance.

We considered 3 alternative multivariate models with progressively more elaborate constraints to be compared with a saturated model: the Cholesky, the independent pathway, and

Table 1. Polychoric Correlation Estimates Calculated Using Best-Fit Results in 712 Twins

	Within-Trait Polychoric Correlations			Cross-Trait Polychoric Correlations					
				Within-Twin			Cross-Twin		
Twin Type	SAD[a]	VASA Score[b]	PD[c]	VASA Score With SAD	VASA Score With PD	SAD With PD	VASA Score With SAD	VASA Score With PD	SAD With PD
Monozygotic (n=349)	0.77	0.52	0.40	0.39	0.40	0.46	0.38	0.36	0.38
Dizygotic (n=363)	0.27	0.19	0.19	0.39	0.40	0.46	0.25	0.17	0.33

Abbreviations: PD, panic disorder; SAD, separation anxiety disorder; VASA, Visual Analog Scale for Anxiety.
[a] Retrospective assessment of *DSM-IV* SAD through direct interview.
[b] After 35% CO_2/65% O_2 inhalation test.
[c] Lifetime occurrence of *DSM-IV* PD.

the common pathway models. For *n* variables, a Cholesky decomposition includes *n* independent genetic and environmental factors. The first factor loads on all traits, the second loads on all traits except the first, the third loads on all traits except the first 2, and so on. The independent pathway model[41,42] predicts that 1 or more common latent genetic and/or environmental factors influence covariation of the observed variables directly (ie, without the mediation of any higher-order factor) and allows the influence of the overlapping factors to differ quantitatively so that the common genetic and environmental factors do not necessarily cause similar groupings of variables. In a common pathway model, covariation is accounted for by genetic and environmental factors through a shared pathway[42]; in this model, a latent intervening variable determined by higher-order latent genetic and environmental factors influences all phenotypes. In terms of life science concepts, such a latent intervening variable could be thought of as 1 or more unifying (patho)physiological mechanisms or systems common to SAD, CO_2 hypersensitivity, and PD, such as a suffocation detector system, as Klein hypothesized.[6]

Although these 3 multivariate models make different assumptions, they all distinguish between common factors that influence all phenotypes and factors specific to each phenotype. While common genetic and environmental factors contribute to explaining the phenotypic covariance, the specific factors explain residual variance not shared by the different phenotypes.

After having identified a best-fitting model to explain phenotypic covariation, we proceeded to test whether the addition of a specified (ie, measured) family-wide environmental agent such as CPL could improve the model's ability to explain covariance. We chose CPL for 2 reasons. First, the classic twin study approach often fails to detect a substantial role for shared environmental agents,[43] but the introduction of specified agents into the models sometimes reveals a contribution of such elements, explaining a proportion of familial aggregation of traits.[32,44] Second, CPL as a specified form of familial environment has been reported to account for 4.9% of total variance in liability to PD.[4]

Preliminarily, we assessed whether the presence or absence of CPL was associated with different parameter estimates, ie, whether its action could to some extent be described in terms of interaction. We calculated the relative fit of a common pathway model (ie, the best-fitting model according to multivariate analyses, see the "Results" section), which allowed the estimates for SAD, post-CO_2 VASA score, and PD to differ across pairs concordant-positive and concordant-negative for CPL against the fit of a simpler model that imposed the same parameter estimates for pairs who had and had not experienced CPL. Then we used our best-fitting model (to which CPL was added as a specified family-shared factor with variance fixed to unity) as a starting point to assess whether (1) early parental loss could further characterize the model by explaining an additional, substantial proportion of variation or covariation, (2) the role of early parental loss could best be described as impinging directly on 1 or more phenotypes (via a residual model whereby CPL directly and differentially influenced the phenotypes), or (3) the role of early parental loss could best be described as impinging on a latent common factor (via a factor model whereby CPL influenced phenotypic covariation via a common, underlying liability or factor shared by all 3 phenotypes).

We based the selection of the model best fitted to the raw data and parameter estimations on a maximum-likelihood approach. The significance of factors was tested by stepwise deletion of variance components in progressively more parsimonious models. Submodels were compared using hierarchical χ^2 tests, as the difference between twice the negative log likelihood (–2LL) for the reduced and the full models have a χ^2 distribution, with *df* given by the difference between the *df* for the 2 models.[45] Models were also compared on the basis of the Akaike information criterion (AIC=–2LL–2 *df*), with the lowest AIC value reflecting a balance between goodness of fit and parsimony.

RESULTS

A full model with the thresholds of all variables (SAD, post-CO_2 VASA score, and PD) allowed to differ across zygosity groups and within twin pairs (first and second twins in a pair) yielded the following: –2LL=2275.15, *df*=2037, and AIC=–1798.85. By applying progressively more elaborate constraints, we observed improvements in parsimony without significant worsening of the fit indices: a model in which the thresholds were constrained to be equal for the first and second twins within a pair and across MZ and DZ pairs yielded the following: –2LL=2303.31, *df*=2055, and AIC=–1806.69. We were able to further reduce the number of parameters via a submodel that estimated 1 cross-twin cross-trait polychoric correlation for each zygosity group and we constrained the within-twin cross-trait correlation to be the same for MZ and DZ pairs[46] and for the first and second twins in a pair (–2LL=2318.36, *df*=2070, and AIC=–1821.64). **Table 1** presents the polychoric correlations for MZ and DZ twins, calculated on the basis of the latter, best-fitting phenotypic model. The size of within-twin phenotypic correlations confirm that the 3 traits covary moderately in our sample. The differences

Table 2. Multivariate Models' Statistics and Comparisons

Model[a]	–2LL	*df*	AIC	χ^2	*df*	*P* Value	Change in χ^2	Change in *df*	*P* Value
Model 1, saturated	2275.15	2037	–1798.85						
Model 2, Cholesky	2324.54	2070	–1815.46	49.39	33	.03			
Model 3, independent	2324.64	2070	–1815.36	49.49	33	.03			
Model 4, common	2325.57	2074	–1822.43	50.42	37	.07			
Model 5, common + $A_C = 0$	2332.51	2075	–1817.49	57.36	38	.02	6.94[b]	1	.01[b]
Model 6, common + $C_C = 0$	2325.57	2075	–1824.43	50.42	38	.09	0.00[b]	1	>.99[b]
Model 7, model 6 + $E_C = 0$	2325.65	2076	–1826.35	50.49	39	.10	0.07[c]	1	.79[c]
Model 8, model 7 + C_{sVASA}, C_{sPD}, $C_{sSAD} = 0$	2325.65	2079	–1832.35	50.49	42	.17	0.00[d]	3	>.99[d]
Model 9, model 8 + $A_{sVASA} = 0$	2327.36	2080	–1832.64	52.21	43	.16	1.71[e]	1	.19[e]
Model 10, model 9 + $A_{sSAD} = 0$	2333.54	2081	–1828.46	58.39	44	.07	6.18[f]	1	.01[f]
Model 11, model 9 + $A_{sPD} = 0$[g]	2327.36	2081	–1834.64	52.21	44	.19	0.00[f]	1	>.99[f]

Abbreviations: A, genetic factor; AIC, Akaike information criterion; C, shared environmental factor; E, unique environmental factor; PD, panic disorder; SAD, separation anxiety disorder; VASA, Visual Analog Scale for Anxiety; –2LL, twice the negative log likelihood.

[a] The subscript C indicates that the influence of the factor is common to the phenotypes, while the subscript S indicates that the influence of the factor is specific, or uncorrelated between phenotypes.

[b] Compared with model 4.

[c] Compared with model 6.

[d] Compared with model 7.

[e] Compared with model 8.

[f] Compared with model 9.

[g] Best-fitting model.

Table 3. Comparison Between Models Implying Different Modes of Influence for CPL

Model	–2LL	*df*	AIC	χ^2_1	*P* Value
Common pathway model[a] + CPL as residual model[b] (model 1)	2645.33	2439	–2232.67		
Common pathway model[a] + CPL as factor model[c] (model 2)	2648.59	2441	–2333.41		
Model 2 + effect of CPL = 0	2660.86	2442	–2223.14	12.27[d]	.001[d]

Abbreviations: AIC, Akaike information criterion; CPL, childhood parental loss; –2LL, twice the negative log likelihood.

[a] Best-fitting model (Table 2).

[b] Childhood parental loss independently influencing the variance of observed phenotypes.

[c] Childhood parental loss influencing the common intervening variable L.

[d] Compared with model 2.

between MZ and DZ cross-twin within-trait correlations are consistent with data showing different degrees of heritability for PD,[32,33] SAD,[5,47] and the acute anxious response to CO_2 stimulation.[19] The greater cross-twin cross-trait correlations in MZ twins compared with DZ twins in turn suggest the importance of genetic factors in explaining phenotypic covariation.

Table 2 presents the results of fitting 3 alternative models compared with a saturated model to explain phenotypic covariation. The common pathway model (model 4) provided a more parsimonious fit than the Cholesky (model 2) and the independent pathway (model 3) models and therefore was selected as the starting point for further refinement. Dropping the common genetic factor from the common pathway model provided a clear and significant deterioration of the fit (model 5), whereas the common shared environmental and the common unique environmental factors could be dropped from the model without significant fit deterioration and with improvement of the AIC (models 6 and 7). Further modeling showed that dropping all specific shared environmental factors (SAD, VASA score, and PD [model 8]), the specific genetic factor for VASA score (model 9), and the specific genetic factor for PD (model 11) did not worsen the model's fit, while a significant deterioration of the model was attained when attempting to drop the specific genetic factor for SAD (model 10). Overall, these analyses show that the best-fitting model is a common pathway model with 1 common genetic higher-order factor, 1 specific genetic variance component for SAD, and specific unique environmental variance components for all 3 phenotypes to explain residual variance.

A common pathway model in which all parameter estimates were allowed to differ across twin pairs who had or had not experienced CPL for all phenotypes yielded –2LL=2286.53 and AIC=–1843.47, whereas a model that equated parameters' estimates across pairs who had or had not experienced CPL yielded –2LL=2288.26 and AIC=–1863.74. We interpret this finding as evidence against interactive (ie, gene by environment) effects of CPL.

Table 3 presents the results of model fitting when CPL is added to the best-fitting common pathway model (model 11) (Table 2), with parameter estimates equated across pairs with or without a history of CPL. The factor common pathway model (model 2) had a better balance between goodness of fit and parsimony than the residual common pathway model (model 1). The added value of CPL to computations is shown by worsening of

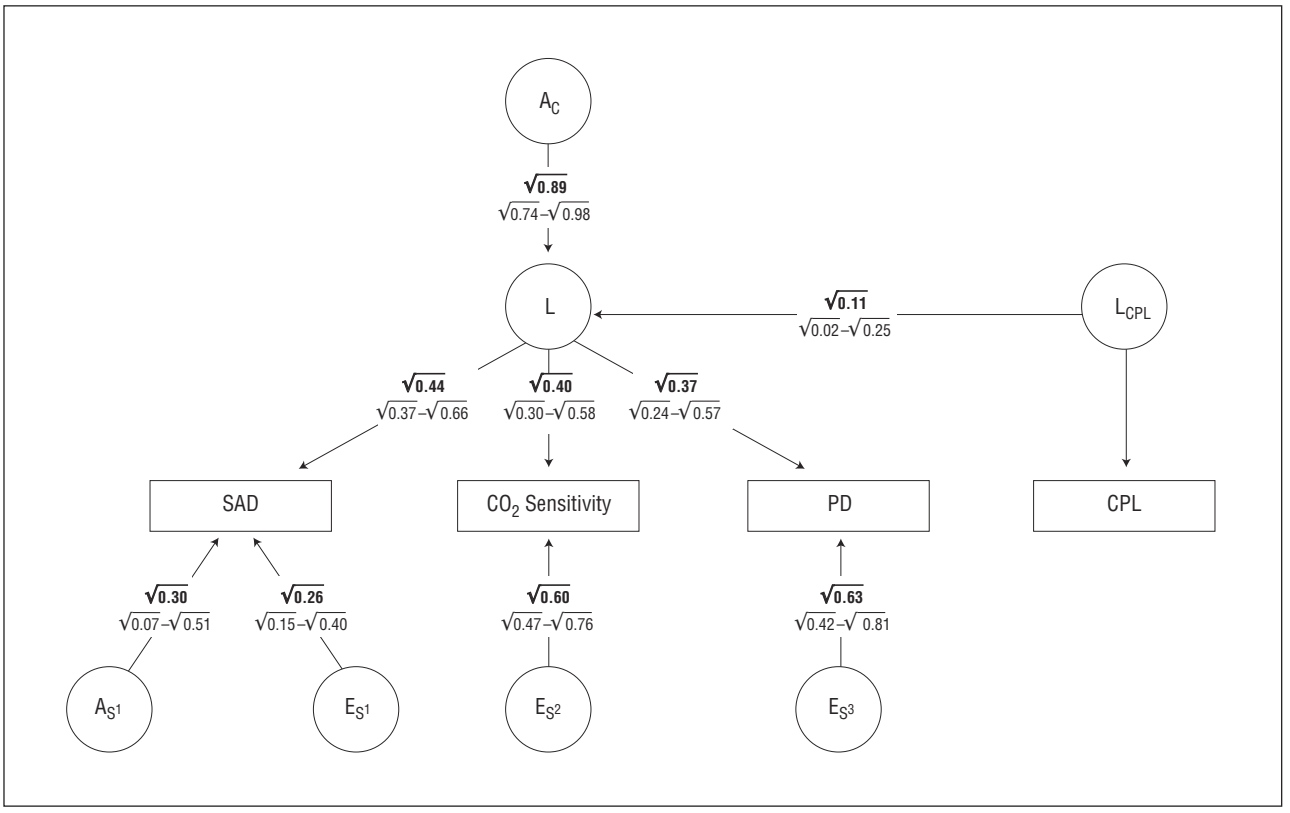

Figure. Best-fitting factor common pathway model with childhood parental loss (CPL) fitted as a shared environmental risk factor and parameters estimates (95% confidence intervals). Values in bold are path values and those beneath are 95% confidence intervals. L indicates common latent intervening variable determined by higher-order latent genetic and environmental factors that influence all phenotypes; PD, panic disorder; and SAD, separation anxiety disorder.

the AIC once this measured risk factor is constrained to 0 (model 3). The **Figure** shows the best-fitting factor common pathway model with CPL fitted as shared environmental risk factor and the parameters estimates.

COMMENT

Our results show that the covariation between separation anxiety in childhood, hypersensitivity to CO_2 (as indexed by the anxiety response to a 35% CO_2/65% O_2 mixture), and PD in adulthood can be explained by a single, shared underlying latent variable influencing the 3 phenotypes. These findings appear to concur with a body of evidence that was collected during almost 5 decades and stemmed from the findings of shared response of PD and SAD to imipramine.[48] Successively, the symptoms of air hunger and the psychobiological trait of sensitivity to lactate and CO_2[6] were pivotal in demonstrating the distinctiveness of PD from other anxiety syndromes and led to the recent formulation of falsifiable models[49] of the neurobiological commonalities linking SAD to PD.

Inasmuch as the hypersensitivity to CO_2 can be considered a relatively specific biological marker,[16,22] our data favor a developmental continuity between SAD in childhood and PD in adulthood, whereby the same underlying neural substrate of excessive sensitivity to a suffocative stimulus[6] appears to act as a bridging element between these 2 anxiety disorders. In turn, genetic effects appear to be the most important underlying cause of such continuity, because the latent variable governing the 3 phenotypes' covariation is largely (89%) influenced by additive genetic determinants, according to our best-fitting model solution.

Like many other studies based on the classic twin study approach, we found that common and specific shared environmental effects could be dropped from stepwise modeling without significant loss of fit. However, as in several other studies, adding CPL (a shared familial factor) to our best-fitting model explained a significant proportion (11%) of the covariation between variables. By multiplying the standardized coefficient paths, one can easily obtain the amount of variance in liability for each phenotype attributable to the additive action of CPL. For instance, in the case of PD, this yields 4.1% (0.11×0.37), a value close to that obtained by a previous study on the effect of CPL on liability for PD in adult women (4.9%).[4] Therefore, inasmuch as CPL can be fully considered to be an aspect of family-wide environment, it helps to explain the familial aggregation of the phenotypes being studied here as well as their covariation within the same individuals.

These results should be interpreted with regard to 7 potential limitations. First, while this is probably the largest sample ever probed for CO_2 reactivity, it is relatively small for structural equation modeling analyses of twin data. Reduced participation rates and relatively small samples, however, remain inevitable constraints of studies that use moderately stressful procedures. Moreover, the use of categorical data and the low prevalence of positive responses to a challenge applied to participants in

the general population somewhat reduces the power of this study. The consequences include the modest precision of several parameter estimates, the relatively wide confidence intervals, and reduced power to effectively choose between alternative multivariate, nested models solutions. Based on the AIC, we can quite safely conclude that CPL is important in explaining the covariation of PD, CO_2 sensitivity, and SAD. There is, however, only a small margin to support the conclusion that CPL exerts a similar proportional effect on all 3 phenotypes through the mediation of the latent intervening variable (ie, the factor model), rather than acting directly and possibly differently on SAD, CO_2 sensitivity, and PD (ie, the residual model). Second, individual response to the 35% CO_2/65% O_2 test appears to be reasonably reliable[31] and stable[50,51] for some but not all PD symptoms. Moreover, while we found that depression and several anxiety disorders did not predict heightened sensitivity to the 35% CO_2/65% O_2 test, we did not measure and could not control for the possible effect of neuroticism, which some[52] found to partially mediate the response to CO_2 stimulation. Third, we did not control for sex effects. Because both PD and heightened reactivity to CO_2 are more common in women than men, the twin correlations for both traits in opposite-sex pairs could be lower than those of same-sex pairs, resulting in artificially increased differences in MZ-DZ correlations. Liability-threshold model approaches to large samples of twins in the general population, however, have not found sex effects on the genetic risk factors for different definitions of PD syndromes.[33] Fourth, the findings are based on the method's assumptions, including the independence and additivity of the latent variables, random mating, and the equal environment assumption. However, regression analyses of the questionnaire items that assessed the degree of environmental closeness between sibs and the possible influence of shared experiences on MZ-DZ twin concordance revealed that these measures of closeness could not predict concordance for either response to the CO_2 test and PD (P=.13-.97)[20] or SAD (P=.21 for DZ twins, P=.87 for MZ twins), suggesting that shared environmental experiences are unlikely to have biased the estimation of genetic covariation between the traits being analyzed. Fifth, this is a partially nonrandomly ascertained sample, but previous controls of the effect of this possible bias on parameters' estimates showed relatively modest effects in our data set.[20] Sixth, each phenotype was assessed at 1 time, which potentially confounds the effects of individual-specific environmental and measurement error, possibly including a recollection bias specific to SAD, which is generally seen in retrospective assessments.[53,54] Seventh, by finding that genetic causes are the main reason for covariation between the studied phenotypes, we partially disagree with 1 study that failed to confirm CO_2 hypersensitivity as a familial risk marker for PD in children and adolescents[14] who were exposed to 5% CO_2 mixtures. However, we are in broad agreement with 5 studies (reviewed by Pine et al[14]) that found greater response to a single breath of 35% CO_2 in adult offspring of patients with PD than in controls. Such inconsistencies may relate to several methodological factors, including the anxiogenic properties of different CO_2 and O_2 concentrations (eg, a 35% CO_2/65% O_2 mixture is simultaneously hypercarbic and hyperoxic) and statistical power issues.[14]

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Unstable Maternal Environment, Separation Anxiety, and Heightened CO_2 Sensitivity Induced by Gene-by-Environment Interplay

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Abstract

Background: In man, many different events implying childhood separation from caregivers/unstable parental environment are associated with heightened risk for panic disorder in adulthood. Twin data show that the occurrence of such events in childhood contributes to explaining the covariation between separation anxiety disorder, panic, and the related psychobiological trait of CO_2 hypersensitivity. We hypothesized that early interference with infant-mother interaction could moderate the interspecific trait of response to CO_2 through genetic control of sensitivity to the environment.

Methodology: Having spent the first 24 hours after birth with their biological mother, outbred NMRI mice were cross-fostered to adoptive mothers for the following 4 post-natal days. They were successively compared to normally-reared individuals for: number of ultrasonic vocalizations during isolation, respiratory physiology responses to normal air (20%O_2), CO_2-enriched air (6% CO_2), hypoxic air (10%O_2), and avoidance of CO_2-enriched environments.

Results: Cross-fostered pups showed significantly more ultrasonic vocalizations, more pronounced hyperventilatory responses (larger tidal volume and minute volume increments) to CO_2-enriched air and heightened aversion towards CO_2-enriched environments, than normally-reared individuals. Enhanced tidal volume increment response to 6%CO_2 was present at 16–20, and 75–90 postnatal days, implying the trait's stability. Quantitative genetic analyses of unrelated individuals, sibs and half-sibs, showed that the genetic variance for tidal volume increment during 6%CO_2 breathing was significantly higher (Bartlett $\chi = 8.3$, $p = 0.004$) among the cross-fostered than the normally-reared individuals, yielding heritability of 0.37 and 0.21 respectively. These results support a stress-diathesis model whereby the genetic influences underlying the response to 6%CO_2 increase their contribution in the presence of an environmental adversity. Maternal grooming/licking behaviour, and corticosterone basal levels were similar among cross-fostered and normally-reared individuals.

Conclusions: A mechanism of gene-by-environment interplay connects this form of early perturbation of infant-mother interaction, heightened CO_2 sensitivity and anxiety. Some non-inferential physiological measurements can enhance animal models of human neurodevelopmental anxiety disorders.

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Introduction

The term 'separation anxiety' applies comprehensively to multiple forms of distress reactions displayed by mammals during postnatal development in conjunction with events of separation from a caregiver [1]. Childhood separation anxiety disorder (SAD) - an extreme human manifestation within this interspecies' propensity - predicts heightened risk for panic disorder (PD) in early adulthood [2], and both PD and SAD [3] share a trait of oversensitivity to higher-than-normal CO_2 concentrations in

inhaled air. Relatively specific responses to CO_2-enriched air mixtures have been described in controlled studies of PD and SAD. These responses consist of both stronger emotional reactions (e.g. panic anxiety), and altered respiratory parameters (i.e., wider tidal volume enhancements and fluctuations, and heightened minute ventilation) [4–6], compared to those seen in control subjects.

Whilst PD is part of the DSM-IV anxiety disorders, the bulk of time-honoured data from clinical observation and empirical research indicates that panic attacks should not be equated with fear responses. Clinical panic attacks are typically spontaneous and unpredictable, and characterised by prominent physical symptoms such as dyspnea, rather than by cognitive symptoms [7]. Another physical symptom, frequently reported during spontaneous and CO_2-provoked panic attacks, is dizziness [8], which may be substantiated in the vestibular dysfunctions often present among people with PD [9]. Endocrinological data contribute to strengthening the view that panic is not a typical emergency fear response. Heightened cortisol levels in spontaneous and CO_2-provoked attacks [10,11] have been found to reflect anticipatory anxiety/individual differences in emotionality, rather than the diagnostic category of PD *per se*. Thus, inasmuch as panic attacks occur in the absence of cues of external danger and are triggered by heightened CO_2 concentrations, they are better seen as inner unconditioned false alarms of biological origin. According to this model, panic attacks derive from a deranged suffocation detector [4] via pathophysiological mechanisms that differ from those underlying general or anticipatory anxiety. Accordingly, most people at the onset of PD are no more anxious/apprehensive/avoidant than people in the general population [12] , and their cortisol levels are within the range of normality [4,13]. However, after having experienced one or more panic attacks, subjects with PD develop a form of avoidance towards places (such as subways or cinemas) where they believe they will experience dyspnea/panic [14], and also become less explorative towards novel, open spaces, behavioural characteristics collectively named 'agoraphobia' [15].

According to twin studies, shared genetic determinants appear to be the major underlying cause of the developmental continuity of childhood SAD into adult PD, and of the association of both disorders with altered sensitivity to CO_2 [16,17]. Moreover, a host of events implying unstable parental environment and separation during childhood (encompassing, e.g, parental military service, job relocation, separation, divorce, death, etc.) can account for a significant additional proportion of the covariation between SAD, CO_2 sensitivity and PD [17]. Thus, in addition to genetic determinants, environmental risk factors affect the liability to these traits, and Ogliari et al. [18] showed that several life events that influence the susceptibility to PD also predict heightened CO_2 reactivity. There is now initial evidence that genetic and environmental determinants may not simply add, but also interact, to influence human responses to CO_2. By modelling the effects of life events in young adult twins, Spatola et al. [19] recently found that adversities that take place within the childhood-adolescence window of risk moderate the genetic variance for CO_2 sensitivity, as assessed by a CO_2 challenge provocation test. Such a form of gene-by-environment interplay is consistent with a diathesis-stress model, and points towards gene-by-environment interactions [20,21] that, while rooted in early life, can exert their effect also in early adulthood.

However, the connections between early perturbations of the offspring-caregiver relationships, separation anxiety, panic, and altered respiratory physiology are still to be clarified.

While the study of human subjects is necessarily limited by the 'natural experiment' approach, the fact that all mammals show similar physiological responses to heightened CO_2 concentrations (i.e., hyperventilation and increased arousal/anxiety) can be exploited to disentangle some of the questions that pertain to the human SAD-PD developmental continuum. By capitalizing on physiological responses to heightened CO_2 concentrations that are relevant to both animal behaviour and the human SAD-PD continuum, one can tackle these questions by fully experimental approaches within the context of animal models [14,22].

Indeed, higher-than-normal environmental concentrations of CO_2 constitute an aversive stimulus for many species. In the C. Elegans [23] and the Drosophila [24,25], CO_2 elicits innate responses of avoidance. In man, heightened CO_2 concentrations induce hyperventilation, subjective air hunger and anxiety [18,26,27] by activating the ventral medulla and subsequently the pons, midbrain, limbic and paralimbic areas, parahyppocampalgyrus, and the anterior insula [28]. Recent data [29] also show that the amygdala is itself a chemosensor that initiates fear responses under hypercarbia and acidosis.

While all mammals respond similarly to the unconditioned suffocative stimulus of heightened CO_2 concentration by increasing ventilation, vigilance and eventually by displaying anxious/avoidant behaviour [30], individuals within the same species differ widely from each other in the intensity of these responses, partially due to genetic factors [8,31,32]. Moreover, different types of early experiences –including environmental adversities not primarily associated with breathing- may affect the plasticity of the mammalian respiratory control system [33].

To sum up the background of this study, seven interrelated points appear fundamental: 1) SAD and PD are on a developmental and pathophysiological continuum, as SAD often precedes PD, and both conditions are associated with CO_2 hypersensitivity; 2) hypersensitivity to CO_2 can be indexed via respiratory parameters and/or exaggerated anxiety responses to heightened CO_2 concentrations in inhaled air; 3) the phenotypes of hypersensitivity to CO_2, SAD, and PD share a genetic background; 4) in man, childhood separation from caregivers/unstable parental environment and early life adversities appear to enhance the risk for SAD/PD/CO_2 hypersensitivity; 5) human responses to heightened CO_2 concentrations may be in part influenced by complex causal mechanisms, whereby the degree of sensitivity to early environmental adversities appears to be under genetic control; 6) rodents are prone to separation anxiety and respond to heightened CO_2 concentrations similarly to man, i.e., by incrementing ventilation and arousal/anxiety; 7) also similarly to man, the increase in ventilation under heightened CO_2 concentrations among rodents yields a degree of interindividual variance, which can be amenable to quantitative genetic estimations.

We speculated that early environmental adversities may moderate a proportion of genetic liability to CO_2 sensitivity through gene-by-environment interplay mechanisms, and that such mechanisms could be substantiated in man and animals. Inasmuch as CO_2 sensitivity represents a valid endophenotype [34–37] that shares part of the liability with human PD and SAD [17], and since CO_2 sensitivity is interspecific, animal models of CO_2 responses can be used as a proxy of a human psychiatric disorder to study gene-environment interplay [38].

To investigate the relationships that link early interference to infant-mother interactions, separation anxiety, and CO_2 sensitivity, we focused on the ventilatory response to heightened CO_2 concentrations in outbred mice repeatedly cross-fostered during the first postnatal days. This approach, largely based on respiratory measurements, permits the circumventing of the difficulties that arise from making inferences about an animal's

emotional state. Moreover, the laboratory context avoids the gene-by-environment correlations that hamper research on gene-by-environment interplay in man.

By implementing a repeated cross-fostering procedure, we sought to address three main questions that pertain to the human SAD-PD developmental continuum: 1) can this form of perturbation of infant-mother relationship alter the pattern of individual reactivity to inhaled CO_2? 2) is the alteration in sensitivity to CO_2 specific and stable? 3) can this type of early manipulation act as an enhancer of individual differences, so that it can reveal mechanisms of genetic control of sensitivity to the environment?

Materials and Methods

Animals

NMRI outbred mice (Harlan, Italy) were used in all experiments. Mice were mated when they were twelve weeks old. Mating protocol consisted in housing two females with one male in transparent high temperature polysufone cages (26.7×20.7×14.0 cm) with water and food available *ad libitum*. Room temperature (21±1°C) and a 12:12 h light dark cycle (lights on at 07.00 a.m.) were kept constant. After 15 days males were removed and pregnant females were isolated in clean cages, and inspected twice a day for live pups. For the first postnatal day (PND0) litters were left with the biological mother.

Postnatal treatment: Repeated Cross-fostering (RCF) Procedure

The Repeated Cross Fostering procedure (RCF) is a new experimental rearing protocol devised to interfere with infant-mother interaction in the first days of life, thus predisposing offspring to separation anxiety without inducing neglect from caregivers. This was based upon the knowledge that when mouse pups are cross-fostered to adoptive lactating dams, they are usually well accepted and nurtured [39], and on the fact that an adoption procedure carried out in the first postnatal days has a low impact on offspring's HPA functioning [40].

Having spent the first postnatal day (PND0) with the biological mother, on PND1 litters were culled to eight pups (4 males and 4 females) and assigned to experimental Repeated Cross Fostering (RCF), or control (CT) treatment. Unlike the 'classical' cross-fostering procedures [41], RCF pups changed caregiver every 24 hours: 4 times in the PND1-PND4 time interval by following a rotation scheme, each dam shifted to 4 different litters and each litter was shifted to 4 different dams (see also Figure S1). The procedure consisted of first removing the mother from the cage, then removing its entire litter, and immediately introducing this litter into the home-cage of a different dam whose pups had just been removed. The RCF pups were then semi-covered with the home-cage bedding of the adoptive mother, which was then reintroduced in the cage and left with this litter for 24 hours. The entire procedure lasted about 30 seconds and took place every day between 10.30 and 11.00 am. This was repeated daily, four times (PND1 to PND4), until reaching the fourth adoptive mother, with which pups remained until weaning (PND0: biological mother, PND1-PND4: adoptive mother 1 to 4- Figure S1). Adoptive dams were lactating females with pups of the same age as fostered litters. This repeated change of caregiver was aimed at interfering with the formation of the infant-mother relationship [42], and to approximate parental instability, a risk factor for internalising disorders, SAD, PD and CO_2 hypersensitivity in man [17,43,44].

Control litters were collected daily and reintroduced to their home-cage, covered with home-cage bedding and had their biological mothers returned within 30 sec, from PND1 to PND4. Animals were weaned when 28 days old and then separated by sex and left in cages with littermates.

Table S1 shows the body weights of RCF and CT individuals during development and in adulthood, as well as their basal body temperature at PND20, measured at a fixed time of day with an infrared body thermometer (153 IRB, Bioseb), in accordance to previously published methods [45].

Maternal Behaviour

Maternal behaviour was observed daily from PND1 to PND7 by an observer unaware of the litter's status (RCF/CT) in two daily sessions (12.00–12.30 and 16.00–16.30), the first session taking place one hour after the cross fostering procedure on PND1-PND4. Maternal behaviour: a) NURSING, including the arched-back and blanket postures, and b) GP/L: grooming and licking pups [46] was monitored with an instantaneous sampling method (one sampling every 2 min), for a total of 16 sampling points/session. The analyses of maternal behaviour were based on the observation of NURSING and GP/L on 10 litters of RCF, and 8 litters of CT pups.

Offspring behaviour

Pups' behaviour was evaluated at: a) PND8, by measuring ultrasonic (USVs) distress vocalizations emitted during isolation, and: b) PND10, by measuring the pups' ability to orient towards and approach maternal/home-cage beddings' [47,48] odour cues (HOMING behaviour, *vide infra*). The assessments of USVs were preceded by transfer of the home-cages into the experimental room at 14.30 of PND8. On PND8, after 1 hour of acclimatization, the mother was removed and transferred into a clean cage, while the offspring was left in the home cage standing on a warm plate set at the temperature of 35,5°C to prevent cooling. Each pup was individually placed for 5 minutes into a beaker containing (i) own-cage bedding (USVs-own) or (ii) clean bedding (USVs-clean) and the vocalizations were recorded. No more than 1 pup/litter/condition was employed and pups were gender-matched for a total of 31 RCF and 44 CT pups. Ultrasonic vocalizations were recorded using an UltraSoundGate Condenser Microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) lowered 1 cm above the top of the isolation beaker containing the pup. The microphone was sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25–140 kHz. It was connected via an UltraSoundGate USB Audio device to a personal computer, where acoustic data were recorded as wav files at 250,000 Hz in 16 bit format. Sound files were transferred to SasLab Pro (version 4.40; AvisoftBioacoutics) for sonographic analysis and a fast Fourier transformation was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. To detect ultrasonic vocalizations, an automatic threshold-based algorithm and a hold time mechanism (hold time: 20 ms) were used. Signals below 30 kHz were truncated to reduce background noise to 0 dB. Inaccurate detections were adjusted manually by an experienced user before running the automatic parameter analysis. The total number of vocalizations emitted in 5 minutes was measured.

The pups' orientation towards familiar odorous cues (HOMING behaviour) was evaluated on PND10 in 32 RCF and 36 CT pups. The assessments of HOMING were preceded by transfer of the home-cages into the experimental room at 14.30 of PND10. The amount of time spent in their home-cage bedding-scented versus (i) clean, or (ii) bedding from an alien dam's cage portions of the apparatus was recorded in 5 minute test sessions. The ability of pups to orient towards familiar odorous cues was evaluated in a

small apparatus (5×33×10 h cm) with a central plexiglas part (5×5 cm, starting point) that separated (with sliding doors) two differently-scented chambers. One of these was covered with pups' home-cage bedding, whilst the other was covered with (i) clean or (ii) home-cage bedding from an alien dam's cage. After 1 minute of habituation in the central part of the maze, the lateral doors were removed and the pups could move freely in the apparatus. The behaviour of pups in the maze was video-recorded for 5 minutes and the time spent in the different compartments was evaluated thereafter. No more than two pups/litter/condition in a gender-matched design were tested in these two HOMING procedures.

Basal Corticosterone levels during development

Dams and pups were sacrificed to measure serum corticosterone basal levels collected via trunk blood samples from 10.00 to 11.00 am. Trunk blood samples were collected after decapitation in RCF and CT offspring (RCF = 16, CT = 14, in a gender-matched design) and their corresponding dams as adoptive or biological mothers (RCF dams = 6, CT dams = 5) before weaning (PND 27–28). After blood centrifugation (20 min, 4°C, 4000 rpm) serum samples were stored at −25°C until assay were conducted. Corticosterone levels were measured using commercially available EIA kits (EIA kit Assay Design, sensitivity 27.0 pg/mL). All corticosterone measures were carried out in duplicate.

Assessments of Face Validity for Human Panic

In order to assess the face validity for human PD/SAD of the current animal model, we capitalised on the following 4 features of human PD, as outlined in the introduction: 1) post-CO_2 cortisol levels do not differ between subjects at heightened risk for PD and control subjects [10,11]; 2) after having experienced one or more panic attacks, subjects with PD avoid places where they anticipate experiencing dyspnea [14]; 3) after having experienced panic attacks, subjects with PD become less explorative towards novel, open spaces and develop agoraphobia [15] 4)vestibular dysfunctions are often described among people with PD [9]. These 4 features of human PD were assessed by proxy among RCF vs. CT adult animals in the 4 following experiments:

1. Corticosterone levels after exposition to CO_2-enriched air Six. The basal serum corticosterone levels obtained from the tail of 6 RCF (2 females 4 males), and 5 CT (2 females, 3 males) individuals (PND70–90) were collected in a litter-balanced design, and compared to corticosterone levels collected in the same individuals by the same tail-cut method after 20 minutes spent an incubator chamber while breathing 6% CO_2-enriched air. As described above, after blood centrifugation serum samples were stored at -25°C until assay were conducted using the commercially available EIA kits (EIA kit Assay Design). All corticosterone measures were carried out in duplicate.

2. Avoidance of CO_2-enriched environments. Place avoidance/preference towards a CO_2-enriched environment was measured in 70–90 day old CT (N = 15) and RCF (N = 11) naïve male mice in a litter-balanced design, in a 'place conditioning' apparatus [49] consisting of two differently-cued chambers connected by a central alley. On day 1 (pretest), the mouse was introduced in the central alley and left free to explore the entire apparatus. During the following 8 days (conditioning), each mouse was confined daily (for 20 min) in one of the two chambers, while the apparatus was introduced into an incubator with either room air or 6% CO_2-enriched air, on alternative days. For each animal, over the 8 training days, one of the two chambers was consistently paired with 6% CO_2-enriched air and the other one with normal air. Testing was conducted on day 10. Animals were placed in the central alley of the apparatus and left free to explore the chambers for 10 min. Pretest and test sessions were videotaped, and subsequently an experienced observer unaware of the treatment conditions recorded the time (in seconds) spent in the different compartments with dedicated software (Smart, Panlab). Place-preference scores were calculated as: [(the amount of time spent in the CO_2-paired compartment)/(amount of time spent in both compartments)] ×100.

3. Effects of CO_2 exposure on exploratory behaviour. Animals (78 individuals, 70–90 PND, gender- and litter-matched) for this experiment were first isolated for 24 hrs in a clean cage with a sliding door, with food and water available. Then subjects were exposed either to room air (RCF: n = 21, CT: n = 20) or to a 20% CO_2-enriched environment (RCF: n = 20, CT: n = 17) for 2 minutes. Immediately thereafter, each animal was allowed to enter a free exploratory apparatus (70×90 cm) connected with the home cage, by leaving the sliding door open for 10 minutes. Each session was video-recorded, and later the percentage of time spent in the centre of the arena (30×50 cm) during exploration of the apparatus was measured by a dedicated software (Smart, Panlab).

4. Evaluation of vestibular function among RCF and CT individuals. Thirty-seven RCF and 32 CT gender and litter-matched individuals (PND70–90) were assessed for their performance at the Rotarod test [50] as a proxy of balance. After training for three sessions in the preceding day, mice had to maintain balance upon an accelerating rotating rod (four trials, from 4 to 40 rpm in 300 seconds), the dependent variable being each subject's latency to fall from the rod [50].

Respiratory Responses

We took into account the following respiratory parameters: tidal volume (i.e. the volume of air displaced between normal inspiration and expiration,TV), respiratory frequency (i.e., the number of breaths an individual takes per minute, f), and minute volume (MV, which is obtained by multiplying TV by f).

On PNDs 16–20, sixty-four pups were tested for their respiratory responses. Thirty-six RCF pups belonging to 14 litters, and 28 CT pups belonging to 12 litters were tested; each subject was exposed to only 1 air mixture condition (air/6%CO_2/10%O_2). For each litter a maximum of 2 pups (one male and one female) were exposed to the same air mixture (air/6%CO_2/10%O_2). We used an unrestrained plethysmograph (PLY4211, Buxco Electronics, Sharon CT) carrying two separate Plexiglass chambers of 450 ml, allowing for the parallel assessment of two animals/session.

Before recording, each subject was closed in the chamber for an acclimatisation of 40 minutes without any air mixture being administered. Subsequently the recording of respiratory parameters started under air condition (baseline) for 20 minutes. Next, the first challenge began: this lasted 20 min and could consist of any of the following three conditions: 1) normal air (20%O_2); 2) 10%O_2, 3) 6%CO_2. A 20 min recovery period (air) followed, then the second challenge (20 min with the same gas mixture as employed in the 1st challenge) took place. A 2nd recovery period (air) of the same duration of the 1st recovery followed, which ended the trial and the recording time. A complete session thus lasted 140 minutes per animal (Figure 1).

Similarly, on PND 75–90, seventy-six adult mice were tested with the same device and procedures, except that only normal air (20%O_2) or 6%CO_2 were employed for adults, given the negative results (see results section) with 10%O_2- and the positive results with 6%CO_2 stimuli in pups. A with the pups, a maximum of 2 adult subjects (one male and one female) per litter were tested

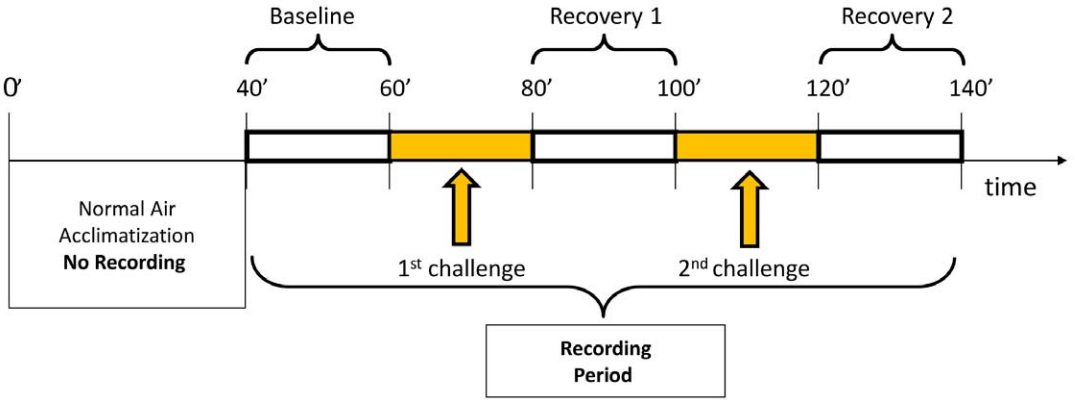

Figure 1. Scheme of the respiratory protocol. During 'baseline' and 'recovery' periods, subjects inhaled normal air. During 'challenge' periods subjects were exposed to one type of air mixture: 6% CO_2-enriched air, or 10%O_2 air, or normal air.
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under one air mixture (air/6%CO_2). Of the 76 adult mice that underwent the respiratory challenges, 33 had previously been exposed to an air/6%CO_2 challenge as pups: for their challenge in adulthood we used the same air mixture they had been exposed to as pups.

Preliminary tests by general linear models were run to assess whether during the first 20 minutes of pre-challenge baseline recording with air, the TV, f and MV parameters differed between RCF and CT pups and adults, and no significant differences were found. Since TV changes are a major physiological strategy to reduce blood P CO_2, we capitalized on the mean percentage of TV increment (ΔTV%) from baseline to air/6%CO_2/10%O_2 as a reference measure to compare animals' respiratory responses to the air mixtures in the experiments. We also preliminarily tested the effects of: weight, sex, and chamber (there were 2 separate plethysmographic chambers) on the respiratory responses of RCF and CT pup and adult subjects separately, by regression procedures. For adult mice, we also tested the effect of previous participation to the challenge as pups. None of these four predictors influenced the respiratory measurements under air/6%CO_2/10%O_2. However, for adult mice, regression of 'previous participation to the respiratory challenge as pup' upon the ΔTV% to 6%CO_2 provided a $r = 0.18$, $F_{1,74} = 2{,}44$, which approached significance: $p = 0.12$. This variable was therefore added as covariate in the analyses of adult respiratory responses.

The effects of treatment (air vs. 6%CO_2 vs. 10%O_2 in pups, and air vs. 6%CO_2 in adults) and postnatal manipulation (RCF vs. standard rearing in CT) in the two exposure challenges were then tested by repeated-measures ANOVA; upon confirmation of significant main effects, differences among individual means were analyzed with post-hoc Tukey's HSD test.

Since the respiratory outcomes in the first and second challenge were highly correlated for all parameters (mean $r = 0.82$, $p = 0.000001$, in pups and adults), for the sake of conciseness we only show the results with the first respiratory challenge in pups and adults.

Quantitative Genetic Investigation of Individual Differences for CO_2 Sensitivity

In quantitative genetics, the variance observed for a given phenotype in a group can be partitioned into a proportion attributable to genetic factors, and another proportion attributable to environmental factors, provided that the degree of genetic relatedness among individuals in the study group is known. Thus, depending on circumstances, one can derive from the phenotypic measurements of human monozygotic and dizygotic twin pairs (additive genetic correlation: 1 and 0.5, respectively), or from the phenotypic measurements of unrelated, half-sib and full-sib animals (additive genetic correlation: 0, 0.25 , and 0.5, respectively), the ratio of genetic variance to phenotypic variance or heritability (h^2) [51]. Gene-by-environment interplay (GXE) mechanisms assume that genetic variance changes as a function of environmental exposure [20,21]. Likewise, in a typical stress-diathesis GXE model, one may observe that the heritability for the trait under study increases as a function of environmental adversities.

To investigate the nature of the proportion of variance of the respiratory response to CO_2 associated with RCF, we crossed 8 unrelated naïve sires with 16 unrelated naïve dams in a 1 sire/2 dam breeding design, yielding litters of 8 pups/dam in full-sib/half-sib degrees of relatedness. Eight litters were exposed to the RCF procedure, while the other eight were reared normally, yielding 64 RCF and 64 CT offspring within a full-sib/half-sib (fs/hs) design [51], whereby for each sire both litters were assigned to the RCF- or CT post-natal condition, as appropriate to conduct variance component analysis, nested ANOVA and heritability estimates [51]. Pups in the fs/hs design were assessed only for respiratory phenotypes.

Animal Care and Statistical Analyses

Unless otherwise specified, all animals took part in only one of the different experiments outlined in this paper, so that they were all naïve individuals. All experiments were conducted under license from the Italian Health Department and in accordance with Italian regulations on the use of research animals (legislation DL 116/92) and NIH guidelines on animal care.

Data were analysed using general linear model approaches, nested ANOVA or variance component analysis, as appropriate.

The variability among dams in providing NURSING and GP/L to fostered (RCF) pups was assessed by two separate ANOVA (Factor: dam) of maternal behaviour collected from PND1 to PND7.

We tested whether the degree of variability in receiving NURSING and GP/L among RCF litters could be assumed as homogeneous by the Levene test (factor : litter).

Similarly, we assessed whether the degree of variability in receiving NURSING and GP/L could be comparable between RCF and CT pups (factor : post-natal treatment) by the Levene test.

Finally, to dissociate the role played by maternal care received by the "final" adoptive mother from PND5 onward from the effects exerted by changes and variation in care received across the 4 cross fostering days (PND1-PND4), we compared the means and variances for NURSING and GP/L in 10 RCF litters by dividing the periods of maternal care into 2 periods of respectively 4 days (PND1–4) and 3 days (PND5–7), as factors.

To ensure sufficient statistical power, the number of subjects in the experiments was determined on the basis of pilot studies carried out in pups and adults. For all figures, bars on histograms indicate standard errors, for all experiments significance was set at $p \leq 0.05$.

Results

Maternal Behaviour

Nursing decreased significantly in time ($F_{6,119} = 3.48$, $p < 0.0035$, Figure 2A) across the PND1-PND7 time span, but neither post-natal treatment, nor time-by-post-natal treatment yielded significant effects (respectively: $F_{1,124} = 0.85$ p = NS; $F_{6,112} = 1.27$ p = NS); consistent with these data, there was no significant difference between RCF and CT mice for weight, measured at different stages of development from PND8 through PND90 (see also Table S1).

Grooming/licking (GP/L) did not vary significantly in time ($F_{6,119} = 1.5$ p = NS, Figure 2B), and neither post-natal treatment ($F_{1,124} = 0.12$ p = NS) nor the interaction of post-natal treatment-by-time yielded significant effects ($F_{6,112} = 1.33$ p = NS).

By ANOVA (factor: dam) we found that NURSING did not differ significantly among dams who took care of the RCF pups ($F_{9,30} = 1.45$ p = NS) as 'adoptive mothers', but GP/L differed significantly among these dams ($F_{9,30} = 2.90$ p = 0.01). However, when we estimated the differences in variance between 10 RCF litters (estimated across 7 days and 4 different dams/litter) for the amount of received care (factor: litter) by Levene test, we found significant differences neither for NURSING (Levene's $_{9,60} = 1.14$, p = NS) nor for GP/L (Levene's $_{9,60} = 1.46$, p = NS). Thus, while there was a certain degree of variability for maternal GP/L towards the fostered pups that was attributable to dams as individuals, the differences in variance of received care among RCF litters from PND1 to PND7 were not significant. This implies that although each RCF litter received care from 4 different dams (i.e., 4 different 'foster mothers') in the PND1-PND4 period, the amount of variability of GP/L and NURSING could be assumed as homogeneous among RCF litters.

Similarly, the total variance of NURSING and GP/L did not differ between RCF and CT pups: when we assessed by the Levene test whether the degree of variability in receiving NURSING and GP/L could be comparable between RCF and CT pups (factor: post-natal treatment), we found no significant differences (NURSING Levene's$_{1,124} = 2.86$, p = NS; GP/L Levene's$_{1,124} = 0.53$, p = NS). This implies that (inasmuch as the variances of NURSING and GP/L could be assumed as sufficient indicators of postnatal treatment) RCF and CT pups were exposed to similar amounts and variability of postnatal care, the only difference between the two types of postnatal treatment being the intrinsic instability of the caregiver.

Finally, when we compared the means and variances for NURSING and GP/L among RCF pups during the PND1-PND4 period, as opposed to the following PND5-PND7 period (factor: PND1–4 vs. PND5–7), we found that the means and variances for both indexes could be assumed as equal (NURSING: Levene's$_{1,68} = 2.43$, p = NS; GP/L: Levene's$_{1,68} = 0.27$, p = NS; NURSING: ANOVA $F_{1,68} = 1.86$, p = NS; GP/L ANOVA $F_{1,68} = 1.03$, p = NS). This implies that within the RCF group, NURSING and GP/L were quantitatively similar when pups were exposed to changing caregivers, and in the following first 3 days of stable motherhood.

Offspring's Behaviour

At PND8, during two different isolation paradigms (conditions: a) 'clean bedding' and b) 'own cage bedding', see also *methods*) we observed an effect of postnatal treatment upon the isolation distress calls in the form of more USVs emitted by RCF pups. Figure 2C (see also caption) shows that there was no 'postnatal treatment-by-condition' interaction. The lower RCF vs. CT difference in isolation distress calls during the 'clean bedding' condition (Figure 2C) may indicate a 'maximum stimulation' effect induced by the absence of any odour in this specific experimental condition. Consistently, a milder stimulation condition (isolation in 'own cage bedding') appeared to unmask the RCF-CT differences more sharply. Sex of subjects did not yield a significant effect, alone or in interaction with the other independent variables.

At PND10, when RCF pups were tested for their preference between own-cage bedding as an alternative to: a) clean bedding, or: b) to an alien dam's bedding (Figure 2D), they showed consistently reduced preference for their own bedding compared to CT pups (Figure 2D). Sex of subjects did not yield a significant effect, alone or in interaction with the other independent variables.

Corticosterone basal levels in RCF and CT Individuals

The mean (±SE) basal serum concentration of corticosterone did not differ in lactating RCF and CT dams (ng/ml: 45.48±17.79 vs 53.02±11.87 respectively; $F_{1,9} = 0.11$, p = NS). Likewise, variance components analyses showed that serum concentrations of corticosterone at PND27–28 did not differ amongst RCF (n = 16 subjects belonging to 4 sibships, n/ml 62.70±3.86) vs. CT subjects (n = 14 subjects belonging to 4 sibships, ng/ml 70.27±4.25; fixed: maternal effect $F_{1,6} = 1.38$, p = 0.28: random: sibship effect $F_{6,22} = 1.36$, p = 0.27).

Assessments of Face Validity for Human Panic (1–4)

1 Corticosterone levels after exposition to CO_2-enriched air. Figure 3A shows that after 20 minutes of 6% CO_2 breathing the serum concentration of corticosterone was heightened in a similar fashion among RCF and CT subjects.

2 Avoidance of CO_2-enriched environments. Adult (PND 70–90) RCF mice exposed to a place conditioning protocol displayed an immediate avoidant response to environments associated with heightened CO_2 concentration, compared to CT mice, as shown in Figure 3B.

3 Free exploratory test after exposition to CO_2-enriched air. A free exploratory test showed that after exposure to 20% CO_2, RCF subjects have a significant reduction of the percentage of time spent at the centre of an arena compared to CT subjects (Figure 3C). After being exposed to room air, on the contrary, RCF and CT subjects did not differ significantly for this behaviour. Sex of subjects did not yield a significant effect, alone or in interaction with the other independent variables.

4 Balance test (Rotarod) in RCF and CT individuals. The performance at the Rotarod shown in Figure 3D was significantly worse among RCF than CT subjects, in that the former showed significantly shorter latency of fall from the rod, which suggests impaired balance. Sex of subjects did not yield a significant effect.

Respiratory Parameters in RCF and CT Individuals

The RCF procedure was associated with higher mean percent increment of tidal volume from baseline (ΔTV%) during 6%CO_2. At PND16–20, the RCF pups showed one-and-a-half times the

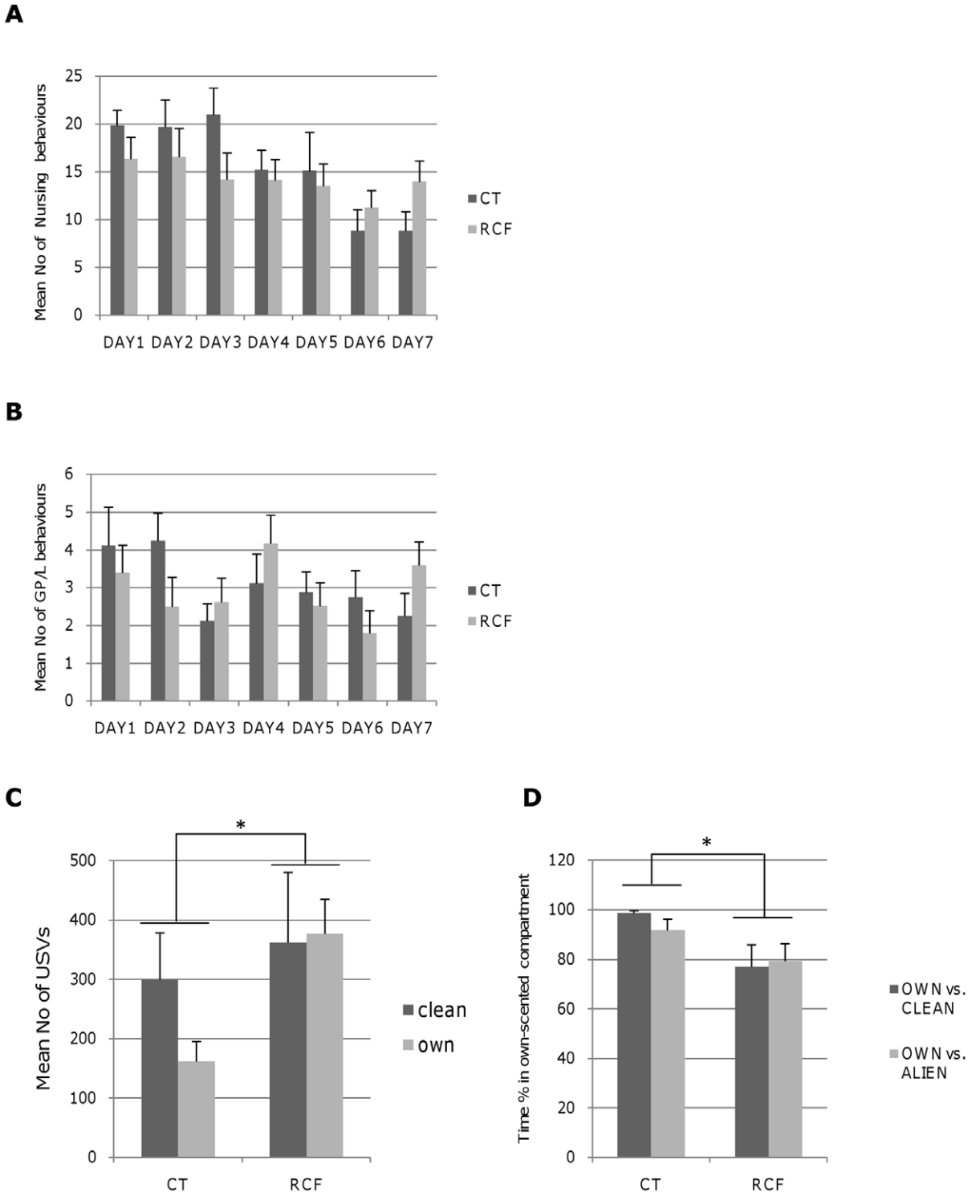

Figure 2. Maternal care and offspring behavioural indices in standard rearing (CT) vs. repeated cross-fostering (RCF) conditions. Sum of 2 daily observations of maternal behaviour: **A**) nursing behaviour, encompassing 'arch-back'+'blanket' postures, and **B**) grooming/licking (GP/L) behaviour towards adoptive (RCF n = 10) and own (CT n = 8) litter, measured during PND1–PND7. Nursing decreased significantly in time, and was comparable in RCF and CT pups across the PND1–PND7 time span. Grooming/licking (GP/L) did not vary significantly in time, and RCF and CT pups received comparable GP/L (see Methods and Results sections for details). Pups' behaviour: **C**) Mean number of ultrasonic vocalisations (USVs) emitted by 8-day old RCF and CT pups. Pups were isolated and exposed for 5' to fresh clean bedding (clean) and own-cage bedding (own). ANOVA showed that the postnatal treatment (RCF vs. CT) yielded a significant effect ($F_{1,73} = 4.24$, $p = 0.04$) while the condition ('clean' vs. 'own' bedding) did not exert a significant effect ($F_{1,73} = 0.84$, p = NS); there was no significant postnatal treatment-by-condition effect ($F_{1,71} = 1.29$, p = NS). **D**) Percentage of time spent during 5 minutes by pups in a compartment containing own-cage vs. fresh clean bedding (own vs. clean), or own-cage vs. an alien dam's bedding (own vs. alien dam). RCF pups spent less time in the compartment with own-cage bedding than controls in both conditions ($F_{1,64} = 7.46$, $p < 0.01$).

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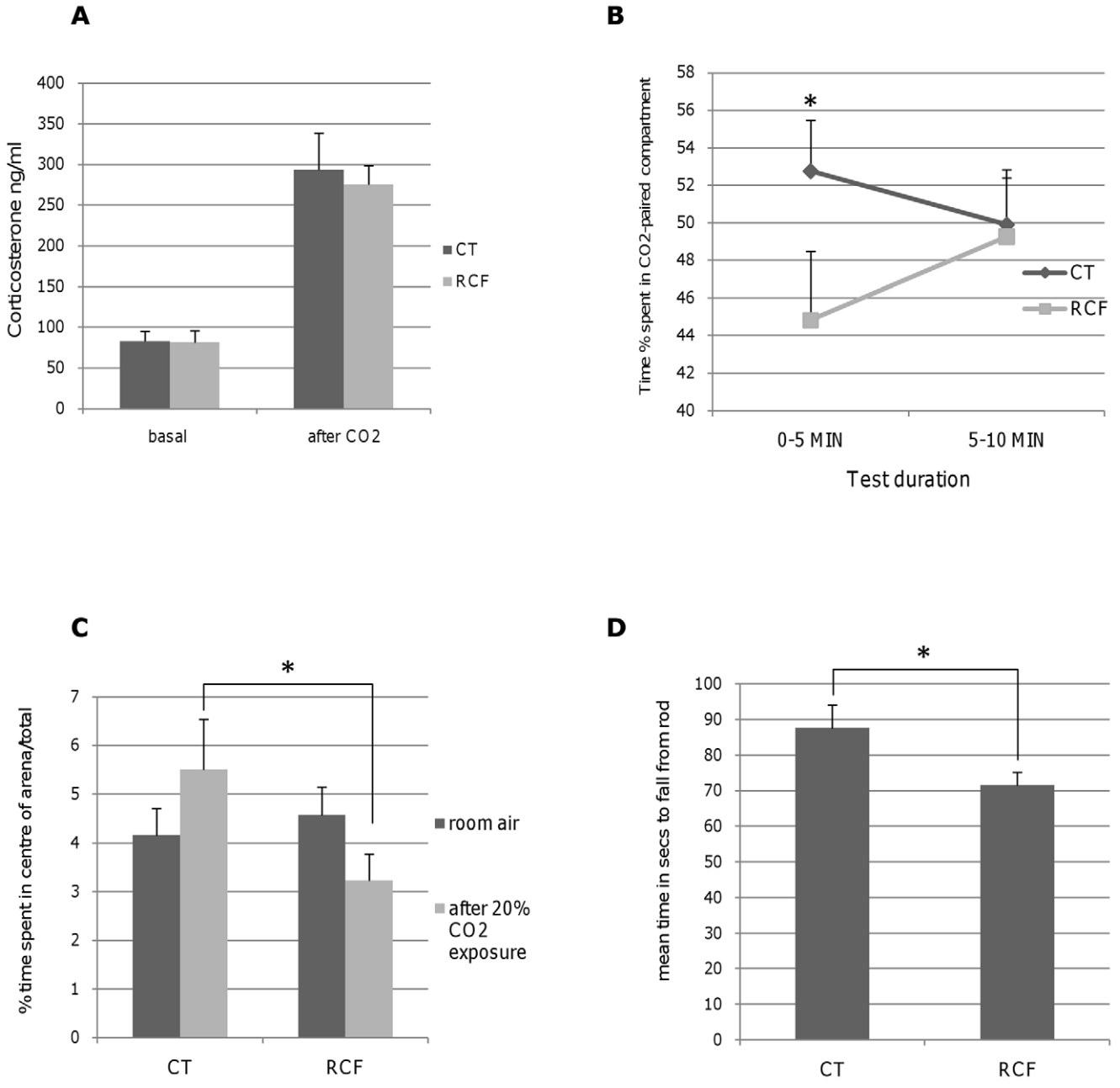

Figure 3. Behavioural and Endocrinological Phenotypes among RCF and CT subjects. A: Serum concentration of corticosterone. In both RCF and CT subjects corticosterone was significantly heightened ($F_{1,9} = 49.71$ $p = 0.0001$) after 20 minutes of 6% CO_2 breathing compared to the basal values obtained in room air breathing; neither 'postnatal treatment' (RCF vs. CT) nor 'postnatal treatment-by-air mixtures' revealed differences for corticosterone serum concentrations (respectively $F_{1,9} = 0.2$ $p = NS$, and $F_{1,7} = 0.08$, $p = NS$). **B**: Place avoidance/preference towards environments with heightened CO_2 concentration (6% CO_2 air mixture): during the first five minutes of the test session, RCF individuals showed significantly higher tendency to avoid the compartment that had been previously paired with 6% CO_2. (ANOVA-R : postnatal manipulation x time interval effect: $F_{1,22} = 4.51$, $p<0.05$, Tukey HSD post-hoc test $p<0.02$). **C**: Free exploratory test. The percentage of time spent at the centre of an arena was significantly influenced by the interaction of postnatal treatment-by-air mixtures ($F_{1,74} = 4.03$, $p = 0.048$) whereby RCF subjects spent significantly less time than CT subjects after exposure to 20% CO_2. Neither the 'postnatal treatment' (RCF/CT), nor the 'air mixtures' (normal air/20% CO_2) variables showed significant effects alone (respectively $F_{1,76} = 1.90$ $p = NS$, $F_{1,76} = 0.13$ $p = NS$). **D**: Latency to fall from the Rotarod. Analysis of variance showed that the performance at the Rotarod was significantly worse among RCF than CT subjects ($F_{1,67} = 5.08$ $p = 0.03$), in that the former showed significantly shorter latency of fall from the rod.
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ΔTV% increase shown by CT pups when they were exposed to 6% CO_2-enriched air mixture, but no difference of ΔTV% in response to hypoxic air (10% O_2), or normal air, compared to CT pups (Figure 4A). The TV increase determined higher MV amongst RCF than CT pups in response to 6%CO_2 (mean MV during 1st 6%CO_2 challenge, respectively: ml/min 53.10±18.22 vs.40.98±12.65, $p = 0.009$), whereas the mean respiratory frequency (f) did not differ significantly in RCF and CT pups during air, 10%O_2, or 6%CO_2 conditions. The RCF procedure also appeared to affect individual sensitivity to 6%CO_2 in a stable way, in that adult mice

(age 75–90 days) that had experienced postnatal RCF, but no other adverse event thereafter, showed higher ΔTV% than CT mice in response to 6%CO_2 (Figure 4B). For both pups and adult mice, the respiratory response to 6%CO_2 was more marked during the 1st challenge than during the 2nd challenge, as shown by a significant effect of 'time' in the general ANOVA-R models, but differences between RCF and CT animals remained consistent and significant within the first- and second challenge recordings.

Individual Differences for CO_2 Responsiveness and Gene-Environment Interplay

Among the unrelated individuals which underwent the respiratory challenges shown in Figure 4, the mean variance of ΔTV% during 6%CO_2 challenges was 57.75 for CT (n = 8) and 124.58 for RCF (n = 13) pups, and: 113.06 for CT (n = 17) and 224.62 for RCF (n = 19) adult subjects (Figure 4). Thus, the RCF procedure appeared to act not only as an enhancer of the mean physiological response to 6%CO_2, but it also acted as a trigger to disclose individual differences for the response to CO_2 amongst unrelated individuals.

On the basis of this datum, and to explore the nature of the proportion of variance of the respiratory response to CO_2 that appeared to be associated with the RCF procedure, we relied on quantitative genetic analyses of data obtained from the full sib/half-sib (fs/hs) design, as outlined in the Methods section.

Table 1 shows the ΔTV% mean increments during 6%CO_2 for $RCF_{fs/hs}$ and $CT_{fs/hs}$ subjects at PND 16–20. According to nested ANOVA, this response to 6% CO_2-enriched air was significantly influenced by both postnatal treatment (RCF vs. CT) and by the degree of genetic relatedness, i.e., a 'sibship' factor. Consistent with this result, Variance Component Analysis showed significantly higher genetic variance (Va) for the ΔTV% response to 6% CO_2-enriched air among $RCF_{fs/hs}$ than $CT_{fs/hs}$ individuals, and almost double heritability, as estimated from half-sibs' correlations [51], for ΔTV% response to 6% CO_2-enriched air among $RCF_{fs/hs}$ compared to $CT_{fs/hs}$ subjects. The 0.21 heritability we found for ΔTV% among $CT_{fs/hs}$ is close to the 0.24 heritability value reported for TV increase under continuous respiration of 7% CO_2-enriched air mixtures in normally-reared rats [52]. The significant difference between the $RCF_{fs/hs}$Va and the $CT_{fs/hs}$Va for ΔTV% response to 6% CO_2-enriched air, and the sizable increase in heritability, indicate the presence of genetic control of sensitivity to the environment [53] evoked by the RCF procedure.

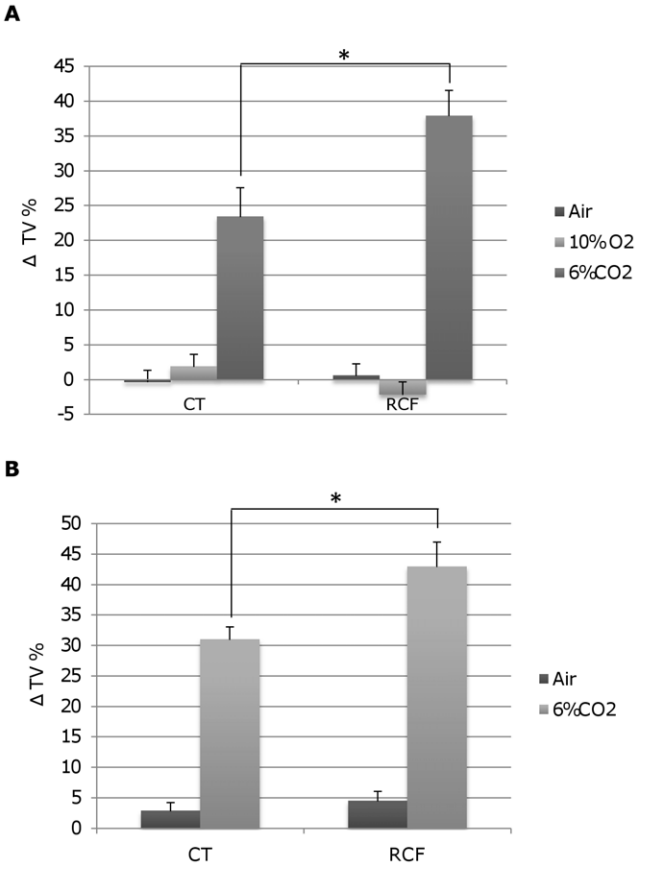

Figure 4. Respiratory responses to air, 10%O_2, or 6%CO_2 in CT and RCF subjects at different ages. Percentage of tidal volume changes from baseline (ΔTV%) for: **a**) 16–20 day-old pups in response to normal air, 10% O_2, or 6% CO_2. The ANOVA-R carried out on two consecutive respiratory challenges (as depicted in Figure 1) indicated a significant effect of: 1) treatment (type of air mixture): $F_{2,58} = 91.30$, $p = 0.000001$, 2) time: $F_{1,58} = 4.34$, $p < 0.05$, and 3) an interaction effect of postnatal manipulation-by-type of air mixture: $F_{2,58} = 9.99$, $p < 0.0002$); Tukey HSD post-hoc test $p < 0.001$; **b**) 75–90 day-old adult mice in response to normal air or 6% CO_2. The ANOVA-R carried out on two consecutive respiratory challenges (as depicted in Figure 1) indicated a significant effect of: 1) treatment (type of air mixture): $F_{1,71} = 184.83$, $p = 0.00001$, 2) time: $F_{1,72} = 35.12$, $p = 0.00001$ and 3) an interaction effect of postnatal manipulation-by-type of air mixture $F_{1,71} = 6.60$, $p = 0.012$). Tukey HSD post-hoc test $p < 0.001$. Sample sizes varied between 9 and 13 animals per group. Only the responses to the first of two consecutive challenges performed for each subject with the same air mixture (air/10% O_2/6% CO_2) are shown in Figure 4 for the sake of conciseness.
doi:10.1371/journal.pone.0018637.g004

Discussion

Our results show that the respiratory reactivity to CO_2-enriched air can be modified by a form of environmental adversity that is not primarily associated with breathing, namely repeated cross-fostering during the first postnatal days. While the RCF protocol used in this study did not interfere with the pups' normal development, as shown by comparable weights and body temperatures in RCF and CT mice, it may have interfered with the formation of infant-mother selective bond. Accordingly, our behavioural data show that while the RCF procedure did not evoke a response of neglect from adoptive mothers, it induced measurable behavioural distress -such as higher number of separation calls, typically interpreted as sign of separation anxiety

Table 1. Tidal Volume percent increment (ΔTV%) in response to 6% CO_2 in RCF and normally-reared (CT) pups at postnatal day 16–20: Mean Values, Genetic Variance, and Heritability figures estimated from unrelated, half-sib and full-sib individuals.

	CTfs/hs	$RCF_{fs/hs}$
Mean ΔTV% response to 6% CO_2-enriched air	34.79±14.63	42.50±17.43*
Genetic Variance for ΔTV% response to 6% CO_2-enriched air	60.01	125.25†
Heritability for ΔTV% response to 6% CO_2-enriched air	0.21	0.37

Fs = Full sibs; hs = Half-sibs.
*Nested ANOVA: 'postnatal treatment RCF vs. CT ' : $F_{1,112} = 8.29$, $p = 0.0048$; 'sibship': $F_{14,112} = 2.17$, $p = 0.01$.
†Bartlett $\chi^2 = 8.3$, $p = 0.004$ by Variance component analysis.
Heritability was calculated on the basis of half-sibs'correlations.
doi:10.1371/journal.pone.0018637.t001

[54]- amongst RCF pups. The RCF also possibly altered the ability to orient and approach maternal cues among these pups. More importantly for the aims of this investigation, the RCF procedure appeared to impact upon sensitivity to 6% CO_2-enriched air selectively (as responses to 10%O_2 and normal air were unaffected by the RCF), and stably from childhood into early adulthood (as responses to 6% CO_2 were similar in pups and in adult RCF subjects). Such specificity of effect conforms with the notion that the regulatory mechanisms of hypercapnic and hypoxic ventilatory responses are functionally separated and genetically dissociated in mice [31]. Moreover, these results point towards an effect of RCF upon the central, more than the peripheral chemoceptors, since the former are much more sensitive to changes in P CO_2 (monitored as $[H^+]$), than changes in P O_2. The 'classical' maternal separation protocol (e.g., 3 hours/day for 10 consecutive days) has been reported to influence the respiratory responses in rats [55]. However these effects are less specific, in that both the responses to hypoxia and CO_2 are altered, and less straightforward to interpret, as opposite patterns of ventilatory response heightened CO_2 have been observed in male and female rats [54]. Unlike what we observed in our RCF mice via the corticosterone data, in rats the 3 hours/day for 10 consecutive days procedure of maternal separation enhances the basal hypothalamic-pituitary-adrenal axis function [56].

The RCF procedure appeared to act not only as an enhancer of the mean physiological response to CO_2. It also acted as a trigger to disclose individual differences for the predisposition to vary the response to CO_2. Our data show significant differences in genetic variance and in heritability between RCF and CT subjects. This indicates that mechanisms of genetic control of sensitivity to the environment are operant here, in the absence of the gene-environment correlations that often complicate the interpretation of heritability variation in man [20,57]. In other words, the RCF procedure brought about a proportion of diversity for CO_2 sensitivity that was ultimately attributable to genetic effects. One may speculate on variations in gene expressions as one likely molecular explanation of our quantitative genetics results, and genomics approaches would now be needed to further explore this paradigm. The results of molecular genetic analyses in this mouse model of separation anxiety could in turn kindle new molecular genetic approaches to human PD and SAD.

Turning to behavioural variables, while the separation calls in RCF and CT pups showed that our procedure evoked more separation anxiety amongst the former [54], it is tempting to relate the avoidance towards CO_2-enriched environments shown by RCF mice to the avoidant/escape behaviour that people with PD display towards crowded, closed environments, where they fear they might experience smothering sensations and panic [14]. Likewise, previous exposure to heightened CO_2 concentrations reduced free exploratory behaviour significantly more among the RCF than the CT subjects.

Two further findings appear to establish a parallel between the RCF mice and humans at heightened risk for the SAD-PD continuum. First, we found similar post-CO_2 corticosterone concentrations among RCF and CT mice, and cortisol levels following spontaneous and CO_2-provoked attacks do not seem to bear strongly upon the diagnosis of PD in man [4,10,11,13]. Second, RCF mice performed significantly worse than CT at the Rotarod. Consistent with this finding, vestibular dysfunctions are found more often among people with PD than among healthy controls [9], with dizziness being frequently reported in spontaneous and CO_2-provoked panic attacks in man [8]. On the other hand, we did not find significant sex-related differences in our respiratory and behavioural tests. This is a possibly relevant discrepancy compared to human data, whereby women typically respond more than men to CO_2 stimulation and have higher prevalence of PD [8].

In conclusion, inasmuch as the genetic determinants that promote overreaction to heightened CO_2 concentrations and naturally-occurring panic attacks in man coincide [16], the adoption of objective respiratory responses, more than the inferential assessment of emotionality or behaviour, is a viable strategy for laboratory animal models of human PD. By these same strategies the developmental pathways of continuity from childhood separation anxiety into adult panic disorder, and the association of both conditions with altered sensitivity to CO_2, can be further clarified, and GXE mechanisms explored in man and animal. Animal laboratory investigations of the mechanisms by which environmental adversities -including childhood unstable parental environment/separation from caregivers- and genetic factors impinge upon CO_2 sensitivity can set a new basis for future, better-tailored genetic approaches to human neurodevelopmental anxiety disorders [20,58]. Our findings further support the investigation of the precise causal mechanisms that connect environmental adversities occurring in sensitive periods of development to health status in childhood and early adulthood [59].

Supporting Information

Figure S1 Scheme of the RCF procedure.
(DOC)

Table S1 Body Weight and Temperature of RCF and CT pups at different developmental stages.
(DOC)

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Author Contributions

Conceived and designed the experiments: MB FRD AM. Performed the experiments: CZ VL RC DO. Analyzed the data: MB FRD CAMS PP-G CZ VL DO. Contributed reagents/materials/analysis tools: TP RV SP-A. Wrote the paper: MB FRD. Revision of the final version of the manuscript: CAMS PP-G VL.

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