Video Article

A Novel Strategy Combining Array-CGH, Whole-exome Sequencing and *In Utero* Electroporation in Rodents to Identify Causative Genes for Brain Malformations

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Abstract

Birth defects that involve the cerebral cortex — also known as malformations of cortical development (MCD) — are important causes of intellectual disability and account for 20-40% of drug-resistant epilepsy in childhood. High-resolution brain imaging has facilitated *in vivo* identification of a large group of MCD phenotypes. Despite the advances in brain imaging, genomic analysis and generation of animal models, a straightforward workflow to systematically prioritize candidate genes and to test functional effects of putative mutations is missing. To overcome this problem, an experimental strategy enabling the identification of novel causative genes for MCD was developed and validated. This strategy is based on identifying candidate genomic regions or genes via array-CGH or whole-exome sequencing and characterizing the effects of their inactivation or of overexpression of specific mutations in developing rodent brains via *in utero* electroporation. This approach led to the identification of the *C6orf70* gene, encoding for a putative vesicular protein, to the pathogenesis of periventricular nodular heterotopia, a MCD caused by defective neuronal migration.

Video Link

The video component of this article can be found at https://www.jove.com/video/53570/

Introduction

The cerebral cortex plays a key role in cognitive and intellectual processes and is involved in emotional control as well as learning and memory. It is therefore not surprising that many neurological and psychiatric diseases result from malformations of cortical development (MCD). The etiology of MCD is complex since both acquired and genetic factors are involved. The cumulative prevalence of genetically determined proportion of MCD is about 2% and they are sporadic in most cases. For instance, the incidence of congenital brain dysgenesis was estimated to

be higher than 1% in the human population, and some forms of MCD are observed in more than 14% of all patients with epilepsy and in 40% of severe or intractable epilepsy[1,2].

Periventricular Nodular Heterotopia (PNH) is one of the most common MCDs and is caused by abnormal migration of neurons from the ventricular zone (VZ) to the developing cerebral cortex. The failure of neurons to migrate results in clusters of heterotopic neurons along the walls of the lateral ventricles which can usually be visualized using Magnetic Resonance Imaging (MRI). The clinical, anatomic and imaging features of PNH are heterogeneous. Nodules may range from small and unilateral to bilateral and symmetric. Common clinical sequelae include epilepsy and intellectual disabilities[3]. Mutations in the *Filamin A* (or *FLNA*) gene, which maps in Xq28, were found in 100% of families with X-linked bilateral PNH and in 26% of sporadic patients[3,4]. A rare, recessive form of PNH caused by mutations in the *ARFGEF2* gene, which maps in 20q13, has been reported in two consanguineous families[5]. Recently, biallelic mutations in genes encoding the receptor-ligand cadherin pair *DCHS1* and *FAT4* have been identified in nine patients affected by a multisystemic disorder that includes PNH[6]. PNH has also been associated with fragile X syndrome[7], Williams syndrome[8], 22q11 microdeletion syndrome[9], duplications at 5p15[10], deletions at 1p36[11], 5q14.3-q15[12], 6p25[13] and 6q terminal deletion syndrome[14,15,16,17,18,19], suggesting that additional causative genes are scattered throughout the genome. However, for approximately 74% of sporadic PNH patients the genetic basis remains to be elucidated[17].

Classical gene mapping approaches such as array Comparative Genomic Hybridization (array-CGH) have proven to be a powerful tools for the detection of sub-microscopic chromosomal abnormalities, however, the genomic regions identified using this approach are often large and contain numerous genes.

The advent of massive parallel sequencing techniques (*i.e.* Whole-Exome Sequencing (WES) and Whole-Genome sequencing (WGS)) has substantially reduced both the cost and the time required to sequence an entire human exome or genome. Nevertheless, interpretation of WES and WGS data remains challenging in the majority of cases, since for each patient tens to hundreds (or even thousands, depending from the type of analysis) variants emerge from data filtering.

To speed up the process of identifying novel MCD causative genes, a novel systematic strategy combining array-CGH, WES and *in utero* electroporation (IUE) screening of candidate genes was designed. IUE allows to selectively inactivate (or overexpress) specific genes or mutations in rodent brains, enabling rapid evaluation of their involvement in corticogenesis[18,19]. RNAi mediated-knockdown or overexpression of one or more candidate genes is expected to cause, when the gene is associated with disease development, localized defects in neuronal migration and/or maturation. Upon the identification of a gene whose inactivation (or overexpression) reproduces the phenotype observed in patients in rodents, it becomes an outstanding candidate for the screening in sporadic patients with MCD. Using this approach, we recently revealed the crucial contribution of the *C6orf70* gene (also known as *ERMARD*) in PNH pathogenesis in patients harbouring 6q27 chromosomal deletions[16].

Protocol

Ethics Statement: Wistar rats were mated, maintained and used in the INMED animal facilities, in agreement with European Union and French legislation.

1. DNA Extraction and Quantification for Array-CGH and WES

1. Extract Genomic DNA (gDNA) from human blood leukocytes from patients using an automated DNA isolation robot or commercially available manual DNA extraction kits according to the manufacturer's protocol. Quantify all samples using a spectrophotometer.

2. Array-CGH Protocol

1. **Restriction Digestion of gDNA**
    1. Double digest 500 ng of purified gDNA from a patient and a commercially available human control DNA with RsaI and AluI for 2 h at 37 °C. Use 0.5 µl of 10 U/µL enzyme for each reaction. Incubate for 20 min at 65 °C to inactivate the enzymes and move the sample tubes to ice.

2. **Sample Labelling**
    1. Add the appropriate volume of Random Primers needed for the microarray format in use to each reaction tube (*e.g.* 5 µl for 1-pack, 2-pack, and 4-pack microarrays). Mix well by pipetting up and down gently.
    2. Transfer sample tubes to a thermal cycler. Incubate at 95 °C for 3 min and then at 4 °C for 5 min.
    3. Prepare one cyanine 3 and one cyanine 5 Labelling Master Mix on ice by mixing the following volumes: 10.0 µl 5X Reaction Buffer, 5.0 µl 10x dNTPs, 3.0 µl Cyanine 3-dUTP or Cyanine 5-dUTP and 1.0 µl Exo(-)Klenow enzyme, 2.0 µl Nuclease-Free Water. Choose the volume of each reagent according to the microarray format in use.
    4. Add the Labelling Master Mix to each reaction tube containing the gDNA. Mix well by gently pipetting up and down.
    5. Transfer sample tubes to a thermal cycler and incubate at 37 °C for 2 h, 65 °C for 10 min and then maintain the samples at 4 °C.

3. **Purification of labelled gDNA**
    1. Add 430 µl of 1x TE (pH 8.0) to each reaction tube.
    2. Place a purification column into 2 mL collection tubes and label the columns appropriately. Load each labelled gDNA onto a purification column.
    3. Centrifuge for 10 min at 14,000 x g in a microcentrifuge at room temperature. Discard the flow-through and place the column back in the 2 ml collection tube.
    4. Add 480 µL of 1x TE (pH 8.0) to each column. Centrifuge for 10 min at 14,000 x g in a microcentrifuge at room temperature. Discard the flow-through.

5. Invert the column into a fresh 2 mL collection tube and centrifuge for 1 min at 1,000 x g in a microcentrifuge at room temperature to collect purified sample.
6. Bring the sample volume to that requested for the microarray format in use using a concentrator or adding 1x TE (pH 8.0). For example, for 1-pack microarray bring the volume to 80.5 µL. Incubate the sample on ice for 5 min and then pipette the solution up and down 10 times.

4. **Preparation of labelled gDNA for Hybridization**
   1. Combine test and reference samples using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample in a fresh 1.5 mL RNase-free tube. For example, for 1-pack microarray ensure that the final volume is 158 µL. Choose the volume of each sample according to the microarray format in use.
   2. Use a spectrophotometer to measure yield, degree of labelling or specific activity by measuring the absorbance at A260 nm (DNA), A550 nm (cyanine 3), and A650 nm (cyanine 5).
      1. Calculate yield using the formula: [DNA concentration(ng/µl) x sample volume (µl)]/1,000 (ng/µg). Calculate Specific Activity using the formula: pmol per µl of dye/µg per µl of gDNA.
         NOTE: For example, for 0.5 µg of input DNA, the yield should be 8 to 11 µg and the specific activity (pmol/µg) should be 25 to 40 for the Cyanine-3 labelled sample and 20 to 35 for the Cyanine-5 labelled sample.
   3. To prepare the amount of Hybridization Master Mix needed for the microarray format in use, mix the following reagents: 50 µL Cot-1 DNA (1.0 mg/ml), 52 µL 10x aCGH Blocking Agent and 260 µl 2x HI-RPM Hybridization Buffer.
   4. Add the appropriate volume of the Hybridization Master Mix to the 1.5 ml RNase-free tube that contains the labelled gDNA to obtain the total volume required for the microarray format in use. For example, for 1 pack microarray, ensure that the final volume for each reaction is 362 µl.
   5. Mix the sample by pipetting up and down, then quickly centrifuge at 14,000 x g and incubate for 3 min at 95 °C and at 37 °C for 30 min.

5. **Chambers assembly and Hybridization**
   1. Load a clean gasket slide into the chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
   2. Dispense hybridization sample mixture onto the gasket well, making sure that the sample is uniformly distributed.
   3. Put a microarray slide down onto the gasket slide assessing that the sandwich-pair is properly aligned. Then, assemble the reaction chamber putting the cover onto the sandwiched slides and hand-tightening the clamp.
   4. Ensure slides wetting by rotating the assembled chamber vertically. Make sure that bubbles are not present in the chamber. If necessary, tap the assembly on a hard surface to move stationary bubbles.
   5. Put the assembled slide chambers in a rotator rack set up to rotate at 20 rpm. Put the hybridization chamber in an oven and perform the hybridization at 65 °C for 24 or 40 h according to the microarray format in use.

6. **Microarray washing and scanning**
   1. Take hybridization chamber out of oven and submerge it in wash buffer 1. Proceed to its disassembly.
   2. Remove the microarray slide and quickly put it into a slide rack in wash buffer 1 at room temperature.
   3. Wash the array slide for 5 min with stirring (use a flea and a magnetic stirrer). Adjust the stirrer speed to a setting of 4 (medium speed), to get good but not too vigorous mixing.
   4. Wash the array slide in wash buffer 2 for 90 s stirring. Gently recover the slide from the buffer (it should emerge dry) and load it into a slide holder with the aid of a slide protector.
   5. Load the slide into the array scanner and perform scanning according to the array manufacturer's instructions.
   6. Analyze the results using the Extraction software provided with the scanner in use according to manufacturer's instructions (V.9.1.3.1).

3. WES Protocol

1. **Target enrichment**
   1. Use the dsDNA BR Assay to determine the concentration of the gDNA sample according to the instrument protocol.
   2. Dilute 5 µg of high-quality double strand DNA with 1x Low TE buffer in a 1.5 mL low bind tube in a total volume of 50 µL.
   3. Set up a sonicator and put a microtube into the loading and unloading station according to the manufacturer's instruction. Keep the cap on the tube.
   4. Slowly transfer the 50 µl DNA sample through the pre-split septa without introducing bubbles.
   5. Shear the DNA according to the settings suggested from the sonicator's manufacturer and assess quality using a Bioanalyzer according to the manufacturer's instruction. The target DNA fragment size is 150 to 200 bp.

2. **Repair of DNA ends**
   1. Prepare the End Repair Reaction Mix by mixing 40 µL of End Repair Enzyme Mix with 10 µL of End Repair Oligo Mix. Mix well on a vortex mixer.
   2. Incubate the samples at 20 °C for 30 min in a thermal cycler and then hold them at 4 °C. Do not use a heated lid.
   3. Add 50 µL of the End Repair Reaction Mix prepared in step 3.2.1 to each 50 µL sheared DNA sample well. Mix well by pipetting up and down or by gentle vortexing.
   4. Purify sample with a beads based purification kit according to manufacturer protocol.

3. **Polyadenylation of 3' ends.**
   1. Add 20 µl of dA-Tailing Master Mix to each end-repaired, purified DNA sample (approximately 20 µL).
   2. Mix well by pipetting up and down or by gentle vortexing.
   3. Incubate the samples at 37 °C in a thermal cycler and then hold it at 4 °C. Do not use a heated lid.

4. **Ligation of the pre-capture indexing adaptor.**
    1. Add 5 µL of Ligation Master Mix to each A-tailed DNA sample.
    2. Add 5 µL of the appropriate pre-capture Index solution to each sample.
    3. Seal the wells and mix thoroughly by vortexing for 5 s. Briefly centrifuge the samples.
    4. Incubate the samples at 20 °C for 15 min in a thermal cycler and then hold at 4 °C. Do not use a heated lid.
    5. Purify the samples with a beads based purification kit according to manufacturer protocol.

5. **Amplification of the indexed library.**
    1. Prepare the appropriate volume of pre-capture PCR reaction mix by mixing 1 µL of Primer Mix and 25 µL of PCR Master Mix. Mix well on a vortex mixer.
    2. Combine 26 µL of the amplification mixture in separate wells of a PCR plate and 24 µL of each indexed gDNA library sample.
    3. Run a PCR program with the following steps: 98 °C for 2 min, 5 cycles at 98 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 10 min. Hold the samples at 4 °C.
    4. Purify sample with a beads based purification kit according to manufacturer protocol.
    5. Assess quality with a Bioanalyzer according to manufacturer protocol.

6. **Pooling of indexed DNA samples for hybridization**
    1. For each capture reaction pool, combine the appropriate volume of each indexed gDNA library sample in one well of a PCR plate. For example, for Human or Mouse All-Exon Capture Libraries, combine 8 libraries per pool using 187.5 ng of each indexed library. Ensure that each final capture reaction pool contains 1,500 ng of indexed gDNA.
    2. Reduce the volume in each well to <7 µL using a vacuum concentrator. Avoid completely drying the sample.
    3. Add sufficient nuclease-free water to each concentrated gDNA pool to bring the final well volume to 7 µL and then vortex the plate containing gDNA vigorously for 30 s. Centrifuge in a centrifuge or mini-plate spinner to collect the liquid at the bottom of the wells.

7. **Hybridization of gDNA library pools to the Capture Library**
    1. To each 7 µL indexed gDNA pool, add 9 µl of blocking Mix. Pipette up and down to mix.
    2. Cap the wells, transfer the sealed plate containing gDNA to the thermal cycler and incubate at 95 °C for 5 min, then hold it at 65 °C. Make sure that the plate is held at 65 °C for at least 5 min.
    3. Prepare the appropriate dilution of RNase Block, on the basis of the size of the capture library (10% dilution for libraries < 3.0 Mb and 25% dilution for libraries >3.0 Mb).
    4. According to the Capture Library Size, combine the proper amount of Capture Library and dilute RNase Block solution in a PCR plate kept on ice. Mix well by pipetting. For capture libraries <3.0 Mb, use 2 µl of Library and 5 µL of RNase Block at 10% dilution. For capture libraries >3.0 Mb, use 5 µl of Library and 2 µl of RNase Block at 25% dilution.
    5. Add 37 µL of Hybridization Buffer to each well containing 7 µL of Capture Library/RNase Block mix. Mix well by pipetting and briefly centrifuge the plate containing the mix.
    6. Maintain the gDNA pool plate at 65 °C while using a multi-channel pipette to transfer the entire 44 µL of Capture Library mixture from step 3.7.5 to each sample well of the gDNA pool plate. Mix well by slowly pipetting up and down 8 to 10 times.
    7. Seal the wells with domed strip caps. Make sure that all wells are completely sealed. Place a compression mat over the PCR plate in the thermal cycler.
    8. Incubate the hybridization mixture for 24 h at 65 °C. Note: Samples may be hybridized for up to 72 h, but must verify that the extended hybridization does not cause extensive evaporation in the sample wells.

8. **Preparation of streptavidin-coated magnetic beads**
    1. Prewarm Wash buffer 2 at 65 °C in a water bath or heat block.
    2. Vigorously resuspend the Streptavidin magnetic beads on a vortex mixer. The magnetic beads settle during storage.
    3. For each hybridization sample, add 50 µL of the resuspended beads to wells of a PCR plate.
    4. Wash the beads and resuspend them in 200 µL of Binding Buffer.

9. **Capture of the hybridized DNA using streptavidin beads.**
    1. Estimate and record the volume of hybridization solution that remains after the 24 h incubation.
    2. Maintain the hybridization plate at 65 °C and use a multichannel pipette to transfer the entire volume (approximately 60 µL) of each hybridization mixture to the plate wells containing 200 µL of washed streptavidin beads. Mix well by slowly pipetting up and down 3 to 5 times.
    3. Cap the wells and incubate the capture plate on a Nutator mixer or equivalent for 30 min at room temperature. Make sure the samples are properly mixing in the wells.
    4. Briefly centrifuge the capture plate in a centrifuge or mini-plate spinner.
    5. Put the capture plate in a magnetic separator to collect the beads from the suspension. Remove and discard the supernatant.
    6. Resuspend the beads in 200 µL of Wash buffer 1. Mix by pipetting up and down until the beads are fully resuspended.
    7. Briefly centrifuge in a centrifuge or mini-plate spinner.
    8. Put the plate in the magnetic separator.
    9. Wait for the solution to clear, then remove and discard the supernatant.

10. **Post-capture Sample Processing for Multiplexed Sequencing**
    1. Prepare the appropriate volume of PCR reaction mixture by mixing the following: 9 µL Nuclease-free water, 25 µL Master Mix and 1 µL Primer Mix according to the number of amplifications. Mix well using a vortex mixer and keep on ice.
    2. For each amplification reaction, place 35 µL of the PCR reaction mixture from step 3.10.1 in the wells of a PCR plate.
    3. Pipette each of the bead-bound captured library pool samples up and down to ensure that the bead suspension is homogeneous.
    4. Add 15 µL of each captured library pool bead suspension to the appropriate PCR reaction mixture well. Mix thoroughly by pipetting until the bead suspension is homogeneous.
    5. Place the plate prepared in point 3.10.2 in a thermal cycler and run the following PCR amplification program: 98 °C for 2 min, 8 to 11 cycles at 98 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Hold the samples at 4 °C.

6. Purify sample with a beads based purification according to manufacturer protocol.
7. Assess quality with a Bioanalyzer according to manufacturer protocol.

11. **Prepare samples for multiplexed sequencing**
NOTE: The final enriched samples contain pools of either 8 or 16 indexed libraries, based on the Capture Library size and resulting pre-capture pooling strategy. The total number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for the Capture Library.
    1. Calculate the number of indexes that can be combined per lane, according to the capacity of the platform. The total number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for the Capture Library. For example, for the Human All Exon v5 library, the Recommended Sequencing Data per Indexed Library is 4 Gb and the Recommended Sequencing Data per Pre-capture Pool is 32 Gb (8-index pools).

12. **Sequence the libraries.**
    1. Load the libraries on the next generation sequencing platform of choice and perform the sequencing run according to the instrument specifications.

13. **Analyze exome sequencing data.**
    1. Run the pipeline and the software of choice for base calling. For example, use Stampy to map reads[20], remove duplicates with Picard (http://broadinstitute.github.io/picard/) and identify single nucleotide polymorphisms and insertion or deletion of bases (indels) with Platypus[21]. Filter synonymous variants and those observed at an allele frequency <5% and compare data with those from parental exomes to identify *de novo* variants.

4. Plasmid DNA Preparation for *In Utero* Electroporation

1. Design several short hairpin RNAs (shRNAs) targeting either the coding sequence or the 3'UTR of the candidate genes as described previously[22].
2. To prevent off target effects test shRNAs specificity by BLAST search against databases using standard methods. Exclude shRNAs displaying more than 50% of complementarity with other rat genes.
3. Clone annealed oligonucleotides into a mU6-pro vector as described previously[23].
4. Purify plasmid DNA with a Maxi-prep according to manufacturer's instructions and make a final concentration of 3 µg/µL.
5. Aliquot 20 µl of DNA (0.5 mg/ml pCAGGS-GFP either alone or with 1.5 mg/ml of shRNA construct) and add 2 µL of Fast green dye (2 mg/mL) to allow visual monitoring of the injection.

5. *In Utero* Electroporation

1. **Surgical procedure**
    1. Anesthetize E15.5 pregnant female rats (E0 was defined as the day of confirmation of sperm-positive vaginal plug), using a combination with a mixture of ketamine/xylazine (0.1 and 0.01 mg per body weight, respectively), which is given via an intraperitoneal injection for anesthetic induction.
    2. Make sure that the animal is fully anesthetized by observing the disappearance of toe pinch reflex.
    3. Shave the animal's lower abdomen using a clipper to remove the fur. Disinfect the skin using scrubs of povidone-iodine and 70% alcohol. Apply veterinarian ointment on eyes to prevent dryness while under anaesthesia.
    4. Place the animal on a heat source and put a sterile drape (with an open window of 4 - 5 cm in the middle) over the incision site. Wear facemasks, lab coat, caps, and sterile surgical gloves. Maintain sterility until the end of the surgery.
    5. Using sharp scissors to make a vertical incision (2 - 2.5 cm) in the skin along the midline in the caudal abdomen and then make an incision of the muscle wall underneath the skin — also of 2.5 cm — along the midline.
    6. Carefully expose one uterine horn from the abdominal cavity. Drop 37 °C pre-warmed normal saline solution to moisturize the embryos. Keep the uterus moist all the time.

2. **Injection of DNA and electroporation**
    1. Gently hold one of the uterine horns with ringed forceps and carefully push one embryo to the uterine wall. Hold the embryo in one hand and with the other hand insert carefully glass capillaries 1 mm from the midline into the left lateral ventricle (2-3 mm deep) and inject approximately 1 µL of DNA with Fast Green to allow visual monitoring of the injection using a microinjector.
    2. Drop normal saline solution on the 3 x 7 mm^2 electrodes surface. Place the positive sterile electrode on the injected side (left lateral ventricle) and the negative sterile electrode on the right ventricle. Deliver 5 electrical pulses at 950 ms intervals with a foot-controlled pedal (4000 mF capacitor charged to 40 V with an electroporator).

3. **Post electroporation procedures**
    1. Put back uterine horns to the abdominal cavity, add drops of normal saline solution to the cavity to allow embryos positioned more naturally and to account for intraoperative fluid loss.
    2. Close the abdominal muscle wall with absorbable surgical sutures, then the skin using a simple-interrupted stitch.
    3. Put the rat back to the cage and monitor the animal until it emerges from the anaesthesia.
    4. For pain management administer buprenorphine (0.03 mg/kg) to the animal by a subcutaneous injection every 8 - 12 h, for up to 48 h.

6. Brain Sample Preparation

1. **Fixation method**
    1. Sacrifice the pregnant rat 5 days after the surgical procedure using gas anaesthesia (isoflurane) followed by a quick cervical dislocation.
    2. Make a vertical incision to re-open the abdominal cavity.
    3. Expose the uterine horns, remove the embryos from the uterus and decapitate them using sharp scissors.
    4. Remove the brain with minimal damage to the tissue: using sharp scissors, make a small incision along the posterior (cerebellum) / anterior (olfactive bulbs) axis of the head to pierce the skin and the skull, then using a pair of forceps peel away the skull to expose the brain and remove it using a small spatula.
    5. Immerse brains overnight at 4 °C in a 4% Paraformaldehyde in 1x Phosphate Buffered Saline (PBS).

2. **Brain sectioning**
    1. Wash brains in 1x PBS for 10 min.
    2. Embed brains in 4% agar in plastic embedding molds.
        1. Prepare 50 mL of 4% agar in 1x PBS for embedding 5 embryonic brains. Ensure that dissolved agarose is at no more than 50 °C.
    3. Keep brains in agarose to polymerize for at least 1 h at 4°C. Remove excess agarose around the brain.
    4. Glue the brain to the vibratome base plate, oriented so the anterior/posterior axis of the brain is perpendicular to the blade. Wait a few minutes for the glue to dry and place the plate with the glued brain into the vibratome chamber.
    5. Fill the vibratome with 1x PBS. Slice 100 µm thick sections.
    6. Transfer brain slices on slides, remove excess liquid, add a few drops of mounting medium and place a coverslip on top of brains

7. Confocal Imaging and Quantitative Analysis

1. Let the mounting medium dry overnight before imaging. Image sections at 10X on a laser-scanning confocal microscope.
2. Using software for quantitative image analysis, convert the image in 8-bit, select one representative GFP positive fluorescent cell and manually define its shape. To do so, click on "Estimate" and draw the border of the cell using the freehand selection tool. In this way the diameter and intensity threshold of the cell are automatically retained by the software.
3. Click on "Find cells" to allow the software to localize transfected cells throughout the cortex. Then, if needed, manually remove false positives.
4. Divide the whole thickness of the cortex into 8 areas of interest normalized in individual sections. To achieve this, click on "Region Tool", select 8 "Region Stripes" where the first (1) and the last (8) stripes correspond to the VZ and to the top of CP respectively. Click on "Apply" to allow the software to count the relative position of GFP transfected cells in the whole cortex. Finally click on "Save Quantification" to export data in an Excel file for the analysis.

Representative Results

The experimental strategy designed to identify novel MCD causative genes is recapitulated in **Figure 1**.

By performing array-CGH in a cohort of 155 patients with developmental brain abnormalities variably combining PNH (**Figure 2A**), corpus callosum dysgenesis, colpocephaly, cerebellar hypoplasia and polymicrogyria associated with epilepsy, ataxia and cognitive impairment, we identified a 1.2 Mb minimal critical deletion in 6q27 shared by 12 patients (**Figure 2B**)[21]. The genomic region contains four known genes (*THBS2*, *PHF10*, *TCTE3* and *DLL1*) and two predicted genes (*WDR27* and *C6orf70*) (**Figure 2B**).

In parallel to array-CGH, WES analysis in 14 patients with isolated bilateral PNH and no Copy Number Variations analysis was carried out, identifying one patient with a *de novo* mutation (c.752T>A: pIle250Asn) in the predicted *C6orf70* gene (Genbank accession number NM_018341.1) (**Figure 3**).

To confirm that the mutation identified in *C6orf70* had a causative role in PNH and to investigate whether haploinsufficiency of the other genes mapping in 6q27 may influence the phenotype, their role *in vivo* on neuronal migration was explored using the *in utero* RNAi-mediated knockdown approach[23,24] to silence their expression in rat cortical neural progenitors. Only genes expressed in the developing rat cortex, *PHF10*, *C6orf70* and *DLL1*, were tested (**Figure 4A**). Different short hairpin RNAs (shRNAs) were generated targeting the coding sequences of these three genes. ShRNAs were then introduced into neuroprogenitors in the rat neocortex by *in utero* electroporation at embryonic day 15 (E15), when neurons committed to upper cortical layers are generated. Green fluorescent protein was used as transfection reporter. Relative distribution of GFP-positive cells was examined 5 days after electroporation. Knockdown of *Dll1* and *Phf10* resulted in a slight delay of radial neuronal migration (data not shown), whereas knockdown of *C6orf70* impaired neuronal migration and gave rise to the development of heterotopic nodules highly reminiscent of those observed in *Flna* knockdown model (**Figure 4B**)[24]. Conversely, transfections with the ineffective *C6orf70* mismatch shRNA had no obvious impact on neuronal migration (**Figure 4B**).

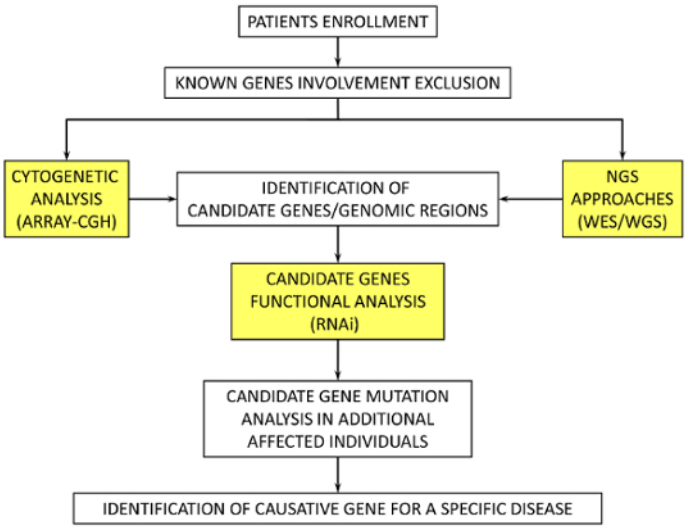

Figure 1: Workflow for the identification of novel MCD causative genes identification. Schematic representation of the different approaches that can be used to identify novel disease causing genes. Boxes coloured in yellow represent the approaches described in the present paper. Please click here to view a larger version of this figure.

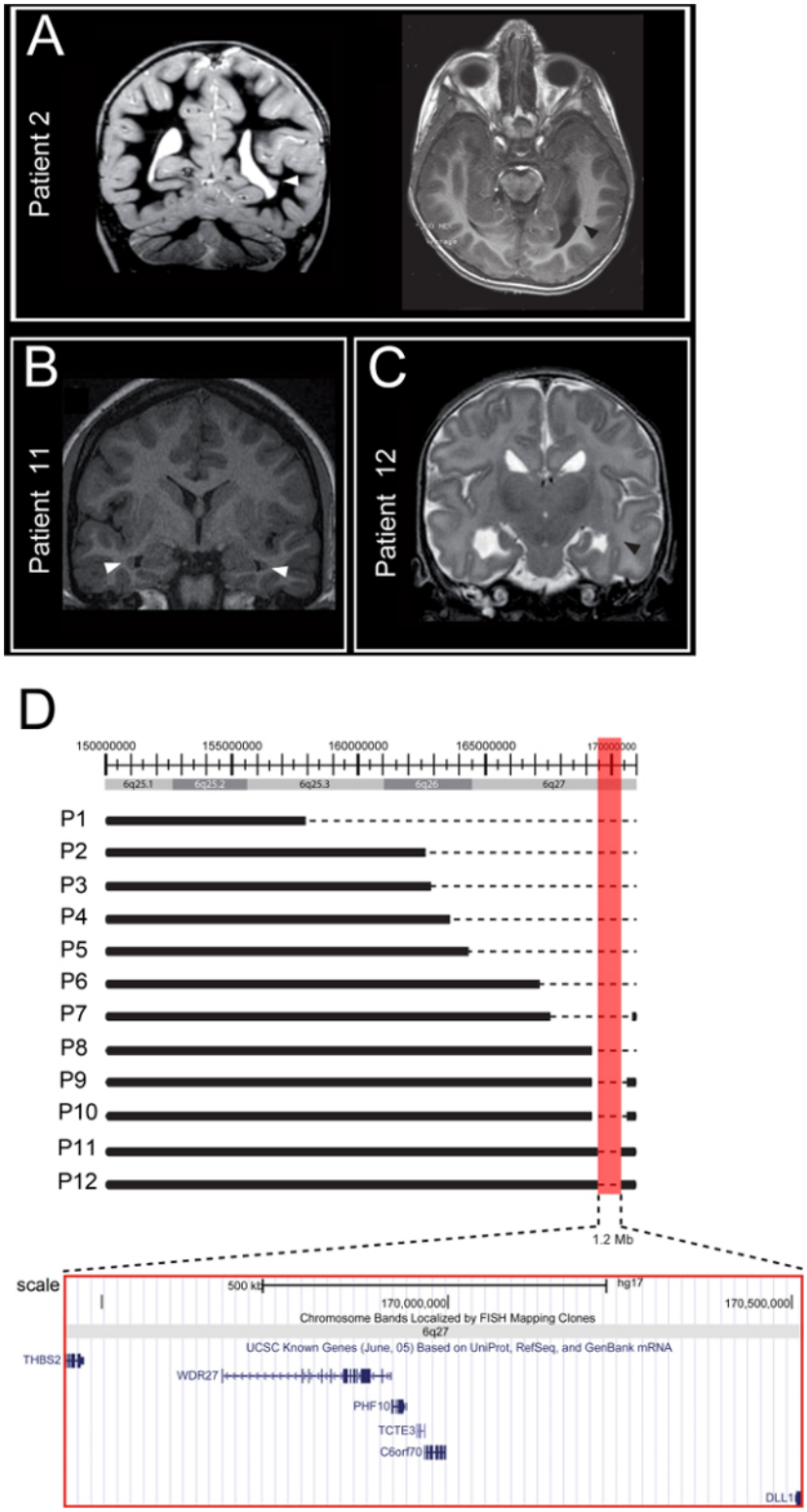

Figure 2: 6q27 deletions cause abnormal brain development with PNH. (**A**) Patient 2. T2-weighed coronal (left panel) and T1-weighed axial (right panel) sections showing PNH lining the wall of the left temporal lobe (white and black arrowhead) below an unfolded insular cortex. (**B**) Patient 11. T1-weighted coronal section, showing bilateral heterotopic nodules along the walls of the temporal horns (white arrowheads). (**C**) Patient 12. T2-weighed coronal section. On the left, PNH is still visible (black arrowhead) and the ventricles are dilated. (**D**) Schematic representation of deletions of the 6q27 region identified in PNH patients using array-CGH (upper part). The horizontal, dashed lines represent deletions identified in patients. The size of the minimal critical deleted region is also indicated and contains four known genes: *THBS2*, *PHF10*, *TCTE3* and *DLL1* and two predicted genes: *WDR27* and *C6orf70* (upper part). Please click here to view a larger version of this figure.

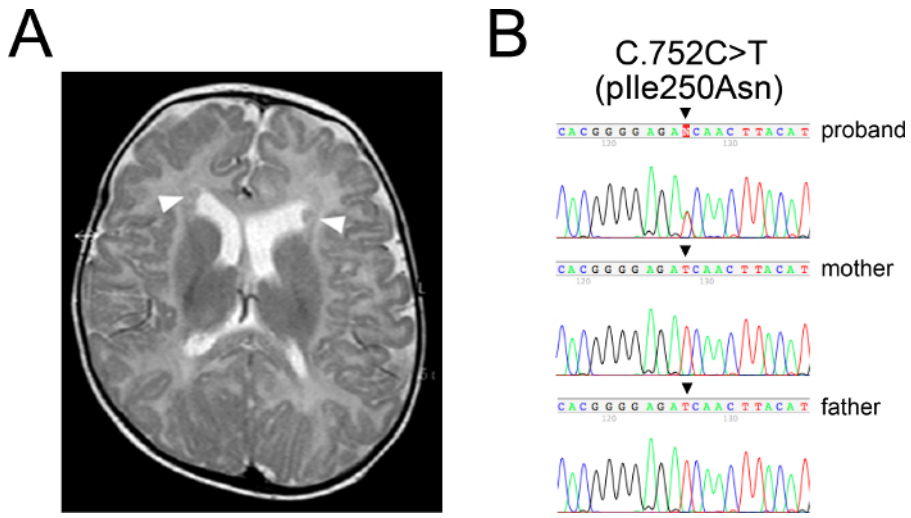

Figure 3: Sequencing results. ( ) T2- weighted axial section showing bilateral PNH in the frontal horns (white arrowheads) in the patient carrying the c.752T>A mutation in C6orf70. (**B**) Sanger sequencing showing that the mutation identified by WES occurred *de novo*. The position of the mutation is indicated by black arrowheads. Please click here to view a larger version of this figure.

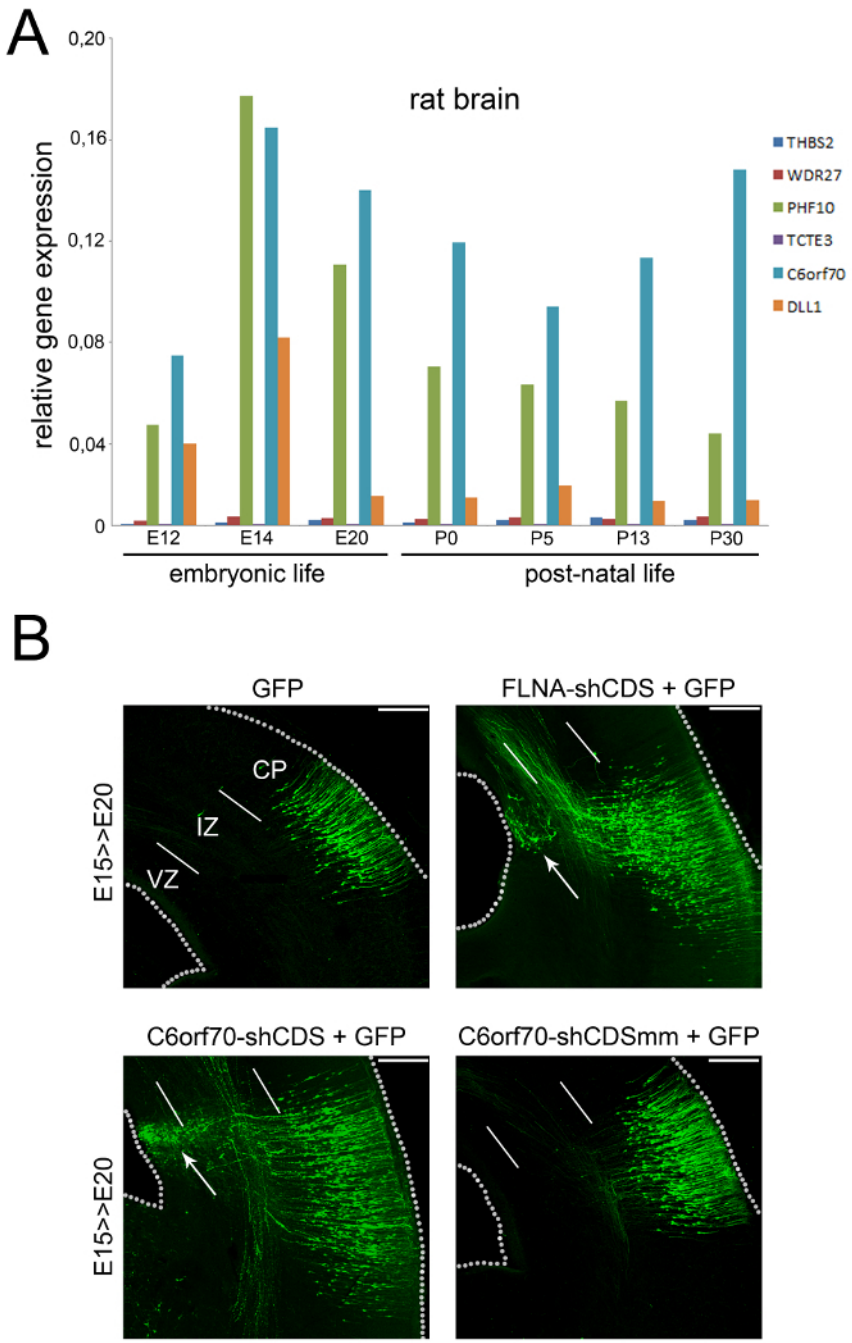

Figure 4: Expression analysis of the genes localized in the 6q27 minimal critical region and C6orf70-knockdown alters radial migration of cortical neurons. (**A**) Quantitative RT-PCR showing the expression of genes contained in the 6q27 critical region in the rat cerebral cortex. Cyclophilin A was used for normalization. (**B**) Representative neocortical coronal sections of E20 rat brains 5 days after electroporation with either Green Fluorescent Protein construct alone (control), or combined with shRNA targeting the *C6orf70* coding sequence (C6orf70-shCDS) or with the relative ineffective mismatch construct (C6orf70-shCDSmm). *Flna*-knockdown (FLNA-shCDS) was used as control of impaired neuronal migration. In utero electroporation with either the C6orf70-shCDS or FLNA-shCDS induced the arrest of GFP-positive cells within the ventricular zone (VZ) (white arrows), whereas the cells expressing GFP alone or in combination with the mismatch construct did not alter neuronal migration. Scale bars = 200 µm. Please click here to view a larger version of this figure.

Discussion

MCDs are important causes of intellectual disability and account for 20-40% of drug-resistant childhood epilepsy[1,2]. Interest in MCDs has increased dramatically over the past decade as a result of two major factors. The first is the improvement in brain imaging (particularly MRI),

which allows physicians and scientists to visualize many brain malformations that were not previously recognized. The other is the evolution of genetic tools that have allowed the identification of many novel MCD causative genes. This has vastly improved our knowledge of the mechanisms that underlie brain development and function, and allowed for more accurate genetic counselling.

In the past, researchers focused their efforts primarily on causative gene identification, leaving the design of functional assays to clarify their role in brain development to later stages. This has become more difficult due to the progression from the study of rare, recurrent genetic disorders to more common sporadic disorders for which traditional gene finding methods are not amenable. Current approaches to identify causative genes often allow the identification of relatively large regions of the genome containing numerous genes (array-CGH) or several variants in a number of candidate genes which are hard to validate (WES or WGS).

Array-CGH and WES (or WGS) approaches have broadened the mutation spectrum for many genetic disorders. Nevertheless, some critical limitations still remain. For instance, array-CGH needs high-quality DNA and fails to identify balanced translocations or small deletions/duplications including those involving one or few exons of a single gene. To overcome this problem, array-CGH may be performed at a higher resolution than conventional probe spacing (*e.g.* using 1M array-CGH kit) or substituted with SNP-array analysis. WES often does not cover large intragenic regions and fails to identify deep intronic mutations. In addition, sometimes the coverage may be too low to identify causative mutations (especially in case of mutations with low percentage of mosaicism). Another critical step for WES is that data analysis and filtering still require a considerable effort. To increase the coverage of WES, the number of patients in a single experiment may be reduced or different capture kits may be used at the same time.

IUE is the most appropriate approach to analyse the impact of genes knockdown on neuronal migration. However, investigations on other steps of cortical developmental, such as neurogenesis and neuronal maturation, are hindered by some technical limitations. Indeed, IUE performed before E13 is often unsuccessful whereas investigations at later stages are restricted by the high rate of postnatal lethality associated to this procedure. In addition, gene-knockdown efficiency may differ among electroporated embryos leading to considerable phenotypic heterogeneity.

Overall, the present protocol has four major critical steps. Although it is not part of the protocol described in the present paper, we have to point out that the first fundamental step to be taken into account is the selection of patients to be enrolled in such studies. Indeed, the process of identifying novel MCD genes requires clinical and imaging investigations in a cohort of patients for whom the phenotype should be as homogeneous as possible. Collecting patients with highly homogeneous phenotype increases the chance of identifying causative mutations in a given gene. However, the minimum number of patients to be enrolled in such studies to achieve success is hard to predict, since it greatly depends on the mutation rate of the different genes. For example, for genes such as *FLNA*, which is mutated in 100% of familial cases of X-linked bilateral PNH and in 26% of sporadic patients, the number of patients needed to identify multiple hits in the gene could be relatively low. Conversely, for genes with low mutation rate, the number of patients to be screened is higher. For example, in the case of *C6orf70,* we were able to identify a single causative mutation only upon screening 64 patients (14 through WES and 50 through conventional Sanger sequencing)[16], estimating a mutation rate for this gene of about 1.5%. The second critical step is the exclusion of mutations in all known MCD genes in order to identify novel causative genes. Thanks to the advent of novel next generation sequencing technologies, mutation screening of known and candidate genes may now be performed in a single experiment. However, appropriate variants filtering should be used to avoid the presence of an excessive number of false positives to be experimentally confirmed and to filter out potential causative mutations. Indeed, the number of candidate genes/variants is particularly high in WES experiments. If the involvement of a given gene is suspected, mutation screening should also be complemented by MLPA analysis, to exclude microdeletions or microduplications. The third critical point is the fact that chromosomal rearrangements are strongly influenced by the location of the breakpoint. In this context, it is worth to exclude, to the greatest possible extent, the disruption or the displacement of cis-regulatory elements distal to genes not included into the deletion/duplication. Finally, *in vivo* RNAi experiments assume that causative genes play a direct role in neuronal migration during embryonic stages. However, the etiology of MCD, including PNH, is heterogeneous and the *in utero* approach could fail to detect the effects of genes involved in developmental steps including neuronal proliferation or cell survival. In addition, the low complexity of the rodent brain could mask the impact of the knockdown or the overexpression of a given candidate gene, which may be more evident in the human brain.

We believe that the integration of genomics and *in vivo* functional studies will help to develop new diagnostic tools for the identification of new MCD causative genes. This strategy could also provide new animal models to test therapeutic targets and understand the pathophysiology of MCD, which have so far been limited by the lack of experimental models and limited access to brain tissue from affected patients. Deciphering the molecular pathways that are associated with MCD disorders will also provide valuable new information about physiological brain development in general.

Disclosures

The authors have nothing to disclose.

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