NeuroImage xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/ynimg

Phenotyping the central nervous system of the embryonic mouse by magnetic resonance microscopy

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ARTICLE INFO

Article history:
Accepted 13 April 2014
Available online xxxx

Keywords:
MRI
Gadolinium
Embryo
Development
Mouse
Brain

ABSTRACT

Genetic mouse models of neurodevelopmental disorders are being massively generated, but technologies for their high-throughput phenotyping are missing. The potential of high-resolution magnetic resonance imaging (MRI) for structural phenotyping has been demonstrated before. However, application to the embryonic mouse central nervous system has been limited by the insufficient anatomical detail. Here we present a method that combines staining of live embryos with a contrast agent together with MR microscopy after fixation, to provide unprecedented anatomical detail at relevant embryonic stages. By using this method we have phenotyped the embryonic forebrain of Robo1/2$^{-/-}$ double mutant mice enabling us to identify most of the well-known anatomical defects in these mutants, as well as novel more subtle alterations. We thus demonstrate the potential of this methodology for a fast and reliable screening of subtle structural abnormalities in the developing mouse brain, as those associated to defects in disease-susceptibility genes of neurologic and psychiatric relevance.

Introduction

Since the advent of transgenesis and the sequencing of the mouse and human genomes, an ever-increasing number of transgenic and mutant mouse strains has been produced aiming to generate mouse models of human conditions, and to understand the underlying biology. Among these, the generation of mutant mice to model human diseases of the central nervous system (CNS) has been particularly widespread (Ross and Walsh, 2001) with a recent emphasis in developmental abnormalities (Manzini and Walsh, 2011; Rakic and Caviness, 1995). A strong case linking a number of neurologic and psychiatric disorders with failures in the early neurodevelopmental program is building upon combined evidence from human and animal studies (Manzini and Walsh, 2011; Ross and Walsh, 2001; Watson and Platt, 2012). Mouse models for these disease-susceptibility genes are of prime importance to elucidate the alterations established early during development and their impact on mature brain architecture.

Structural phenotyping of the brain conventionally relies on histological techniques. While these techniques are perfectly suited to analyze cellular and subcellular architectural details of brain anatomy and connectivity, they are extremely time-consuming when meso- and macroscopic evaluations of brain structure are required. Furthermore, methodological artifacts during sample processing inherent to the technique, such as tissue shrinkage or defects in tissue sections (folding, braking, irregular staining, etc.), profoundly affect the reliability of assessments, most particularly volumetry and 3D arrangement of structures (Cleary et al., 2011a). Alternative methods for three-dimensional imaging of mouse embryos have become available more recently, including optical projection tomography (Sharpe et al., 2002), block-face microscopy (Denk et al., 2012; Rosenthal et al., 2004), micro-computed tomography (Kulandavelu et al., 2006) and light sheet fluorescence microscopy (Tomer et al., 2012). Effective, non-invasive and high throughput methods are needed for the rapid and accurate phenotypic analysis of mouse mutant brains.

Magnetic resonance imaging (MRI) has been increasingly used to advance these efforts and characterize brain phenotypes of mouse mutants. Different imaging protocols for small rodents have been developed and its utility demonstrated both in normal and mutant strains (Ahrens et al., 1998; Cahill et al., 2012; Lee et al., 2005; Lerch et al., 2011; Silva et al., 2008; Zhang et al., 2010). In addition, in the last decade MRI has begun to be used in developmental studies, to phenotype the developing mouse at embryonic stages (Chuang et al., 2011; Petiet et al., 2008; Szulc et al., 2013). MRI offers a number of advantages over traditional histological sectioning, particularly its ability to generate, in a non-invasive manner, three-dimensional and non-distorted datasets that allow the accurate calculation of morphometric parameters and, hence, to identify novel phenotypes.

Of the various applications of MRI in morphometry and structural phenotyping, imaging of the mouse embryo brain is clearly lagging

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http://dx.doi.org/10.1016/j.neuroimage.2014.04.043
1053-8119/

behind and there is much potential that remains unfulfilled. If developmental neuroscience has so far benefited little from MRI, this is likely because of the poor tissue contrast and small size of the embryonic brain structures as compared to the adult brain. However, using systems of high magnetic field strength and active staining with contrast agents, enhancing structures not normally visible on MRI, allows image resolutions in the microscopic range (15–30 μm) (Cleary et al., 2011a; Norris et al., 2012; Petiet et al., 2008). Indeed, applying the so-called MRI-microscopy and Gadoteriol as contrast agent (CA), the first non-destructive atlas of the whole mouse embryo has been contributed (Petiet et al., 2008). Although these works represent a tremendous advance in MRI of the mouse embryo, the level of anatomical detail attained so far for the developing CNS only allows differentiating gross morphological subdivisions.

Here we report a method for MRI microscopy that yields outstanding anatomical detail of the embryonic mouse CNS at relevant neurodevelopmental stages. The method is fast, accessible and versatile, which makes it ideal for rapid and quantitative screening of neurodevelopmental phenotypes. Central to the method is the staining of living embryos with the CA gadolinium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Gd-DOTA) which provides selective contrast enhancement to the developing CNS. We show the results obtained with different variations of the staining protocol, including a convenient in utero staining of the embryos through intraperitoneal (i.p.) administration of Gd-DOTA to the pregnant mother. Furthermore, we have identified the specific brain tissues being predominantly highlighted by Gd-DOTA staining, thus clarifying the anatomical correlate of contrast enhancement. As a proof of concept we have applied this technique to phenotype the forebrain of mouse embryos double mutant for the axon guidance receptors Robo1 and Robo2, and found not only the well-known structural defects that characterize this mutation but also new, more subtle defects. Taken together, the characteristics of our methodology demonstrate its potential to identify, with a fast and accessible protocol, subtle structural abnormalities in the developing brain, as those thought to be associated with defects in disease-susceptibility genes with psychiatric relevance (Courchesne et al., 2007; Levitt et al., 2003; Paul et al., 2007).

Material and methods

Animals

Animals were handled in accordance with the UMH IACUC, following Spanish and European regulations. Mouse embryos at gestation day 14.5 (E14.5, n = 3) and E17.5 (n = 8) were on an ICR genetic background. Robo1/2 double mutant E17.5 embryos (n = 4) were obtained by mating double transgenic Robo1$^{+/-}$, Robo2$^{+/-}$, which were maintained in CD1, C57BL6, and mixed CD1-C57BL6 backgrounds. Genotyping was performed as described previously (Long et al., 2004).

Contrast agent administration

Animals were injected with Gd-DOTA (Dotarem®; Guerbet, Aulnaysous-Bois, France) at 0.5 M in four different ways: (i) 25 μl/g of Gd-DOTA was injected in the peritoneum of the pregnant mother 2 h before fixation of the pups, (ii) 1.5 to 2 μl of the Gd-DOTA was injected in the lateral ventricle of the embryo 30 min. prior to fixation, (iii) 5 μl/g of Gd-DOTA was injected in the peritoneum of the embryo 30 min. prior to fixation and (iv) 5 μl/g of Gd-DOTA was added to the paraformaldehyde (PFA) used to perfuse the animals. Embryos were kept submerged in ice-cold dissection buffer to maximize their survival and minimize any possible pain until fixation. E14.5 mouse embryos were fixed by immersion in phosphate-buffered 4% PFA for 3 h at 4 °C. E17.5 embryos were perfused transcardially with the same fixative solution (including Gd-DOTA only in protocol (iv), see above). Briefly, each individual embryo was immobilized, the thoracic cavity cut open and the beating heart exposed, the right atrium cut open, and a 30-gauge needle was inserted into the left ventricle, through which the fixative solution was manually injected into the heart and bloodstream for 5 min. Post-fixation of E17.5 embryos was performed by immersion in phosphate-buffered 4% PFA for 3 h at 4 °C. After fixation, the whole embryo or the dissected head was extensively washed with phosphate-buffered saline 3 times before embedding in 4% low melting agarose for MRI imaging. Samples were conserved at 4 °C until the imaging session (ranging between 1 to 3 days).

MRI

Experiments were carried out in a horizontal 7 T scanner with a 30 cm diameter bore (Biospec 70/30v, Bruker Medical, Ettlingen, Germany). The system had a 675 mT/m actively shielded gradient coil (Bruker, BGA 12-S) of 11.4 cm inner diameter. A ^{1}H rat brain receive-only phase array coil with integrated combiner and preamplifier, no tune/no match, in combination with the actively detuned transmit-only resonator (Bruker BioSpin MRI GmbH, Germany) was employed.

Three types of images were acquired for embryos covering the brain (Figs. 2–6), whole head (Figs. 1H–K) or the complete body (Figs. 1A–G). Phantoms containing embryo head or whole body in agarose were placed in a custom-made holder and positioned fixed in the magnet isocenter. T_2 weighted anatomical images were collected using a rapid acquisition relaxation enhanced sequence (RARE), applying the following parameters: field of view (FOV) 10 × 10 mm, 15 slices, slice thickness 0.8 mm, matrix 128 × 128, effective echo time (TE_{eff}) 56 ms, repetition time (TR) 2 s and a RARE factor of 8. The B0 field distribution in a large voxel (30 × 30 × 30 mm^3) containing the volume to be imaged was acquired (FieldMap). Samples were localized with a T_2 weighted RARE sequence, and first- and second-order shims adjusted with MAPSHIM application in a sufficiently large voxel containing the brain. 3D data were acquired using a RARE 3D sequence with TR 1000 ms, TE_{eff} 45 ms, BW 75000 Hz, RARE factor 16 and 26 averages in a total acquisition time of 24 h. Antialiasing was implemented to avoid image folding (P1 and P2 of 1.2 and 1.1, respectively). For brain studies, the matrix size was 230 × 270 × 150 using a FOV of 7.6 × 9.0 × 5.0 mm^3, which yielded an isotropic resolution of 33 μm in 24 h acquisition time. For full head imaging, a FOV of 9.70 × 11.8 × 7.6 mm^3 with a matrix size of 234 × 286 × 184 was used, rendering an isotropic resolution of 41 μm, with 26 averages in a total acquisition time of 30.75 h. Finally, whole embryo imaging was performed with the same protocol in a FOV of 24.0 × 15.5 × 10.8 mm^3 with a matrix of 300 × 194 × 134, rendering an isotropic resolution of 80 μm. In this last case, 10 averages were acquired in 4.5 h.

Image processing quantification

Data were acquired and processed with a Hewlett-Packard console running Paravision 5.1 software (Bruker Medical GmbH, Ettlingen,

Fig. 1. Gd-DOTA-enhanced MRI in the whole embryo selectively highlights the central nervous system (CNS). (A–C) Visualization of a whole E17.5 mouse embryo with an i.p. injection of Gd-DOTA, in external view (A), a single MRI plane (B), and the full stack of MRI images (C). Gd-DOTA treatment selectively highlights CNS structures such as the spinal cord (SC), mesencephalon (Mes) and telencephalon (Tel), and to a lesser extent other structures like the stomach (Sto), nasal cavity (nc), mouth (mo) and trachea (tr). (D–G) Full MRI image stacks filter-processed to further distinguish body organs with different contrast levels. This filtering allows to exquisitely reveal the outer contours of the CNS (shades of gray) over all other body organs and tissues (shades of red). (F, G) Detailed views of the forebrain from the dorsal and ventral sides illustrating the three-dimensional reconstruction of the neocortex (NCx), olfactory bulb (OB) and basilar pons (bp). (H–K) Rotation and re-slicing of the brain volume to generate example 2D-planes in horizontal (H), coronal (I), sagittal (J) and oblique (K) orientations. Inserts in the top left corner show the position of the selected slices over the 3D reconstructed brain. Arrowheads in (K) point towards the thalamo-cortical pathway. Scale bar: 3 mm (A–E), 1.3 mm (H–K).

Please cite this article as: Martínez-Martínez, M.A., et al., Phenotyping the central nervous system of the embryonic mouse by magnetic resonance microscopy, NeuroImage (2014), http://dx.doi.org/10.1016/j.neuroimage.2014.04.043