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Repeated exposure of the developing rat brain to magnetic resonance imaging did not affect neurogenesis, cell death or memory function

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ABSTRACT

The effect of magnetic fields on the brain is a matter of debate. The objective of this study was to investigate whether repeated exposure to strong magnetic fields, such as during magnetic resonance imaging (MRI), could elicit changes in the developing rat brain. Embryonic day 15 (E15) and postnatal day 14 (P14) rats were exposed to MRI using a 7.05 T MR system. The animals were anesthetized and exposed for 35 min per day for 4 successive days. Control animals were anesthetized but no MRI was performed. Body temperature was maintained at 37 °C. BrdU was injected after each session (50 mg/kg). One month later, cell proliferation, neurogenesis and astrogenesis in the dentate gyrus were evaluated, revealing no effects of MRI, neither in the E15, nor in the P14 group. DNA damage in the dentate gyrus in the P14 group was evaluated on P18, 1 day after the last session, using TUNEL staining. There was no difference in the number of TUNEL-positive cells after MRI compared with controls, neither in mature neurons, nor in newborn progenitors (BrdU/TUNEL double-labeled cells). Novel object recognition was performed to assess memory function 1 month after MRI. There was no difference in the recognition index observed after MRI compared with the control rats, neither for the E15, nor for the P14 group. In conclusion, repeated exposure to MRI did not appear to affect neurogenesis, cell death or memory function in rats, neither in late gestation (E15–E18) nor in young postnatal (P14–P17) rats.

1. Introduction

The effect of magnetic fields on the brain is a matter of debate. It has been reported that prenatal exposures to LTP-patterned magnetic fields produced quantitative alterations in neuronal density and affected behavior [1]. Other studies showed that magnetic fields had no effects on DNA damage [2], cell proliferation, migration or fetal development [3]. Transcranial magnetic stimulation is used clinically for patients with movement disorders and stroke [4,5]. Cortical neuronal cultures exposed to strong static magnetic fields showed that low intensity fields stimulated cell proliferation and high intensity fields induced extensive cell death [6]. It is only in magnetic resonance imaging (MRI) that humans are regularly exposed to strong static magnetic fields. Effects of strong magnetic fields on whole organisms have been found to be either absent or transient [7].

Neural stem and progenitor cells persist and continue to proliferate mainly in the hippocampal dentate gyrus (DG) and the forebrain subventricular zone (SVZ) [8]. Cell proliferation and differentiation are regulated by physiological [9,10] and pathological [11] stimuli and can be modified pharmacologically [12]. It has been reported that, for example, ionizing radiation [13] and anesthesia [12] can affect neural stem and progenitor cell proliferation and differentiation in the brain negatively, and that enriched environment [14] and physical exercise [9] have positive effects. It has been reported that hippocampal neurogenesis is associated with recognition memory [15]. The purpose of this study was to determine whether clinically relevant levels of exposure to MRI might have any effects on the developing brain. This is the first report to our knowledge investigating the effects of MRI on neuronal stem and progenitor cell proliferation, differentiation and death.

2. Materials and methods

2.1. Animals

Time-mated 15-day pregnant Wistar rats, carrying embryonic day (E15, two-thirds gestation) embryos, or postnatal day 14 (P14) pups were purchased from B&K Universal AB (Sollentuna,

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Sweden). Rats were anesthetized with isoflurane (1.7%) (Isoba, Schering-Plough Corporation, NJ, USA) in a mixture of air and oxygen (1:1). Pregnant rats (n = 3/group) or P14 pups (n = 6/group) were subjected to MRI for 35 min daily, 4 days in a row. Controls were anesthetized only. BrdU was injected intraperitoneally (50 mg/kg) immediately after the 35 min of anesthesia. The body temperature of the rats was kept at 37 °C using a heating pad. All animal experiments were approved by the Ethical Committee of Gothenburg (application No. 05-212).

2.2. Magnetic resonance procedure

Magnetic resonance (MR) measurements were performed on a 7.05 T Bruker BioSpec MR system (Bruker Biospin GmbH, Ettlingen, Germany) equipped with a superconducting magnet (Magnex Scientific, Oxford, UK) with a size of the room temperature bore of 21 cm. A volume coil with 72 mm inner diameter (Bruker Biospin, Ettlingen, Germany) was used for transmission of radiofrequency and a quadrature receiver coil (Rapid Biomedical, Rimpar, Germany) adapted to the geometry of the rat head was used in receive mode. The animal was placed on an electronically heated animal bed (Rapid Biomedical, Rimpar, Germany) and kept at 37 °C. First, a pilot scan with three orthogonal slices (field of view 5 cm, slice thickness 1 mm, matrix 128^2) was acquired by a FLASH sequence (TE 4 ms, TR 100 ms, flip angle 30°) using a hermit pulse (pulse length 1000 μs, 47.5 mW). Total image time was 51 s 200 ms. Next, high resolution images (field of view 2.4 cm, matrix 256 × 192) in 40 slices (slice thickness 0.5 mm, slice distance 0.5 mm) were acquired using a multi-slice multi-echo sequence (TR 1790 ms, TE 20 ms). The sequence uses a 90–180° hermit pulse combination with a pulse length of 3000 μs at 6.94 mW (90°) and 1860 μs at 44.12 mW (180°). Six measurements were averaged for data acquisition in a total measurement time of 34 min and 33 s.

2.3. BrdU administration

The thymidine analog bromodeoxyuridine (BrdU) (5 mg/ml, dissolved in 0.9% saline) (Roche, Mannheim, Germany) was injected intraperitoneally, 50 mg/kg, immediately after every MR or anesthesia session. All animals were sacrificed at P45 (Figs. 1A and 2A).

2.4. Immunohistochemistry

The animals were deeply anesthetized with phenobarbital and perfusion-fixed with 5% formaldehyde in 0.1 M PBS, followed by immersion fixation in the same fixative overnight. One hemisphere was dehydrated with graded ethanol and paraffin-embedded, the other hemisphere was kept in fixative with 30% sucrose solution. Sagittal vibratome sections (30 μm) were stored in tissue cryoprotectant solution (25% ethylene glycol, 25% glycerol and 0.1 M phosphate buffer) at −20 °C.

BrdU immunolabeling was performed on free floating vibratome sections. BrdU was exposed through DNA denaturation as follows: incubation in 2 M HCl for 30 min at 37 °C, neutralization in 0.1 mol/L borate buffer (pH 8.5) for 10 min and several rinses in Tris-buffered saline (TBS; 0.08 mol/L Tris–HCl, 0.016 mol/L Tris–Base, 0.15 mol/L NaCl, pH 7.5). Sections were blocked in 3% donkey serum in TBS for 30 min and then incubated with primary antibody (monoclonal mouse anti-BrdU IgG, 1:400, Boehringer Mannheim) in blocking solution for 16 h at 4 °C. After rinsing with TBS, sections were incubated for 2 h at room temperature with secondary antibody (biotinylated donkey anti-mouse IgG, 1:1000, Jackson ImmunoResearch Lab, West Grove, PA, USA). After rinsing in TBS, the staining was visualized with avidin-peroxidase for 1 h (ABC-kit, Vectastatin Elite, Vector Laboratories) followed by 26.5 mg/mL diaminobenzidine, with 0.01% H_2O_2 and 0.04% NiCl.

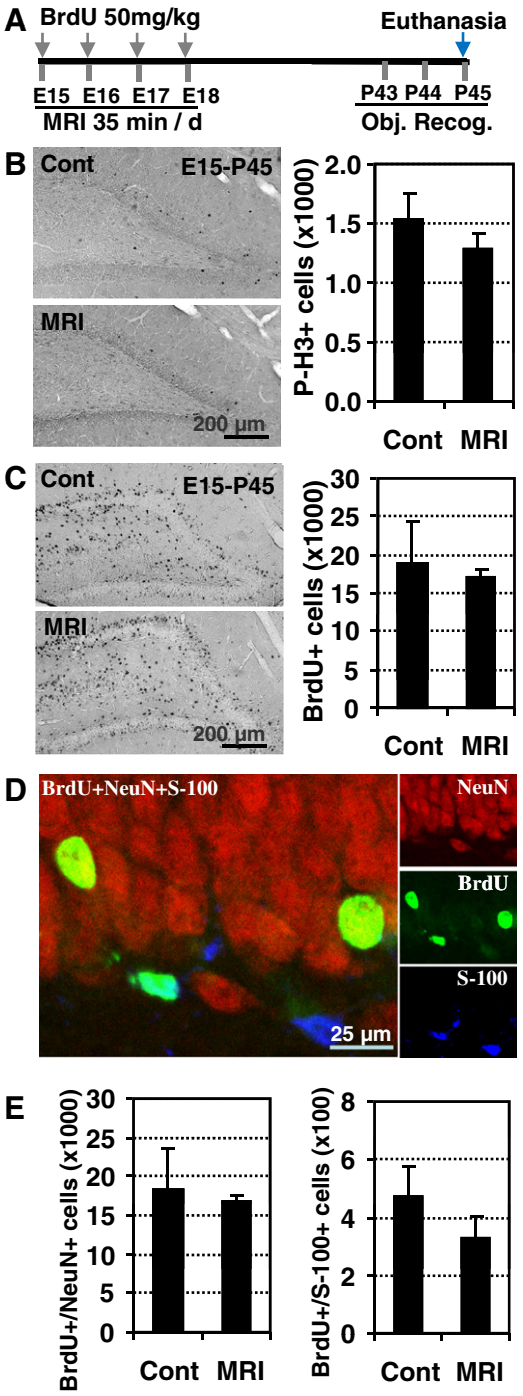

Fig. 1. Cell proliferation and neurogenesis in the E15 rat hippocampus after MRI. (A) The experimental paradigm, where pregnant dams were subjected to MRI and injected with BrdU daily from embryonic day 15 (E15) to E18. Object recognition testing was performed from postnatal day 43 (P43) to P45. (B) Representative phospho-histone H3 (P-H3) stainings, a marker of proliferation, on P45. The number of P-H3-positive cells was not different between the groups (n = 10 controls and 6 MRI). (C) Representative BrdU stainings from P45. The number of BrdU-labeled cells in the DG was not different between the control and MRI groups (n = 10 controls and 6 MRI). (D) A confocal image showing triple labeling of BrdU (green), NeuN (red; neurons) and S-100β (blue; astrocytes) in the DG. (E) The bar graphs show the numbers of BrdU/NeuN and BrdU/S-100β double-positive cells, respectively. There were no differences between the groups (n = 10 controls and 6 MRI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

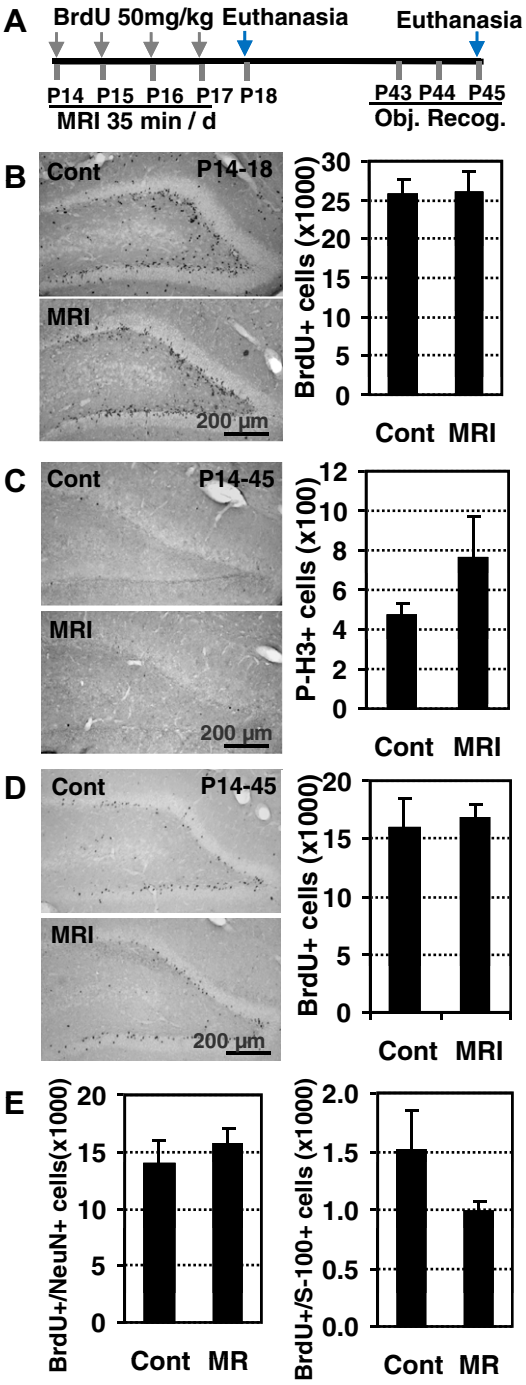

Fig. 2. Cell proliferation and neurogenesis in the P14 rat hippocampus after MRI. (A) The experimental paradigm, where rat pups were subjected to MRI and injected with BrdU daily from postnatal day 14 (P14) to P17, followed by euthanasia on P18. Object recognition testing was performed on a separate group of animals from postnatal day 43 (P43) to P45. (B) Representative BrdU stainings 24 h after the last MRI as a measure of proliferation. The number of BrdU-labeled cells in the DG was not different between the control and MRI groups (n = 6/group). (C) Representative phospho-histone H3 (P-H3) stainings on P45, a marker of proliferation, on P45. The number of P-H3-positive cells was not different between the groups (n = 6/group). (D) Representative BrdU stainings on P45 showing the scattered labeled cells. The number of BrdU-labeled cells was not different between the groups (n = 6/group). (E) The bar graphs show the numbers of BrdU/NeuN and BrdU/S-100β double-positive cells, respectively. There were no differences between the groups (n = 6/group).

For the phospho-histone H3 staining, the sections were denatured in 10 mM sodium citrate solution, pH 9.0 for 30 min at 80 °C and endogenous peroxidase activity was removed using 0.6% H_2O_2 for 30 min. After rinsing, the sections were blocked with 3% donkey serum and then rabbit anti-phospho-histone H3 polyclonal Ig G (1:200, 5 μg/ml, Upstate, Lake Placid, NY, USA) in blocking solution for 16 h at 4 °C. After rinsing with TBS, sections were incubated for 2 h at room temperature with secondary antibody (biotinylated donkey anti-rabbit IgG, 1:1000, Jackson ImmunoResearch Lab, West Grove, PA, USA).

The phenotypes of BrdU-labeled cells were determined using triple immunofluorescent staining. Antibodies against NeuN and S-100β were used to detect mature neurons and astrocytes, respectively. After antigen recovery and blocking as above, sections were incubated with rat anti-BrdU (1:250, 2 μg/ml; clone: BU1/75, Oxford Biotechnology Ltd., Oxfordshire, UK), mouse anti-NeuN monoclonal antibody (1:200, 5 μg/ml; clone: MAB377, Chemicon, Temecula, CA, USA), rabbit anti-S-100β (1:1000; Swant, Bellinzona, Switzerland) in PBS at room temperature for 60 min. After washing, the sections were incubated with secondary antibodies, Alexa 488 donkey anti-rat IgG (H + L), combined with Alexa 555 donkey anti-mouse IgG (H + L) and Alexa 647 donkey anti-rabbit IgG (H + L) at room temperature for 60 min. All secondary antibodies were from Jackson ImmunoResearch Lab, and were diluted 1:1000. After washing, the sections were mounted using Vectashield mounting medium.

DNA strand breaks were detected using TUNEL labeling (Roche, Mannheim, Germany). Following deparaffinization, rehydration and antigen retrieval, sections were incubated with 3% bovine serum albumin in 0.1 M Tris–HCl (pH 7.5) for 30 min followed by 50 μl of TUNEL reaction mixture (terminal deoxynucleotidyl transferase, fluorescein-dUTP and deoxynucleotide triphosphate) on each sample for 60 min at 37 °C in a moisture chamber. After washing, the sections were incubated with 4% donkey serum for 30 min and then incubated with mouse anti-BrdU for 60 min, followed by Alexa 555 donkey anti-mouse at room temperature for 60 min. After washing, the sections were mounted using Vectashield mounting medium.

2.5. Cell counting

The numbers of BrdU-labeled cells and phospho-histone H3-positive cells were counted in the granule cell layer (GCL), including the subgranular zone (SGZ), using unbiased stereological counting techniques. All cells were counted on every 12th section by using a semiautomatic stereology system (StereoInvestigator, MicroBrightField Inc., Magdeburg, Germany) as described previously [12]. For the immunofluorescence staining, at least 50 BrdU-positive cells in the DG were phenotyped using a confocal laser scanning microscope (Leica TCS SP, Heidelberg, Germany) and the ratio of Brdu/NeuN and BrdU/S100β double-labeled cells was calculated for each sample. The total number of newborn neurons (BrdU/NeuN-positive) and astrocytes (BrdU/S100β-positive) in each sample was calculated based on the number of BrdU-positive cells and the ratio of double labeling. The total number of neurons in the GCL was assessed by counting the neuronal nuclei stained with DAPI, using stereological counting techniques.

2.6. Object recognition

Object recognition was tested on rats in an open field plastic arena measuring 65 × 48 × 28 cm with bedding material covering the floor, in a room with ventilation and background noise, by an investigator blinded to the treatment of the animals as described previously [12,16,17]. Data were expressed as recognition index (the difference between the time spent exploring the novel and the familiar objects, corrected for the total time exploring both objects). A recognition index of zero means that the animal spent

equal time exploring the two objects and a positive recognition index indicates that more time was spent exploring the novel object.

2.7. Statistical analysis

All data were expressed as mean ± SEM Student's unpaired *t*-test was used to compare GCL volumes, BrdU- and P-H3-positive cell numbers, numbers of newborn neurons and astrocytes between as well as the total numbers of neurons in the GCL between the two groups. The Mann–Whitney *U* test was used to compare the recognition index between the two groups. Significance was assumed when $p < 0.05$.

3. Results

3.1. Cell proliferation and differentiation

To evaluate the effects of MRI on cell proliferation and differentiation in the hippocampus, E15 dams received BrdU injections once daily for 4 consecutive days and male pups were euthanized on P45 (Fig. 1A). Cell proliferation as indicated by P-H3 staining was not different between the control ($n = 10$) and MRI ($n = 6$) groups (Fig. 1B). The newly generated, surviving cells were scattered, as indicated by BrdU labeling on P45, but were not different between the control and MRI groups (Fig. 1C). Quantification of neurons and astrocytes born from E15 to E18, as judged by BrdU/NeuN and BrdU/S-100β co-labeling (Fig. 1D), revealed no difference between the groups (Fig. 1E).

P14 male pups were injected with BrdU once daily for 4 consecutive days and euthanized either on P18 or P45 (Fig. 2A). Short term effects on proliferation were evaluated 24 h after the last MRI by quantification of BrdU labeling. BrdU-positive cells were detected in clusters in the subgranular zone (SGZ) of the DG and the numbers were not different between the control and MRI groups (Fig. 2B). Long term effects on proliferation were evaluated on P45 by quantification of phospho-histone H3 (P-H3) immunostaining. The number of P-H3-positive cells was not different between groups (Fig. 2C). Newly generated, surviving cells, as indicated by BrdU labeling on P45, was not different between groups (Fig. 2D). Quantification of neurons and astrocytes born from P14 to P17, as judged by BrdU/NeuN and BrdU/S-100β co-labeling, revealed no difference between the groups (Fig. 2E).

3.2. Cell death in the dentate gyrus after MRI

To test if repeated MRI could induce DNA damage, TUNEL-positive and TUNEL/BrdU double-positive cells were counted in the

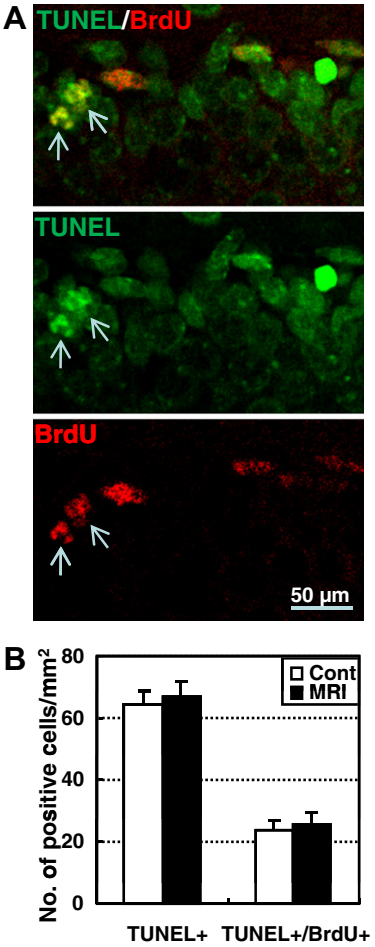

Fig. 3. Cell death in the granule cell layer 24 h after the last MRI of P14 rats. (A) A confocal image showing double labeling of BrdU (red) and the DNA damage marker TUNEL (green) in the granule cell layer (GCL) and the subgranular zone (SGZ). (B) Quantification of the number of TUNEL-positive cells as well as TUNEL and BrdU double-positive cells revealed no differences between the control group and MRI groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

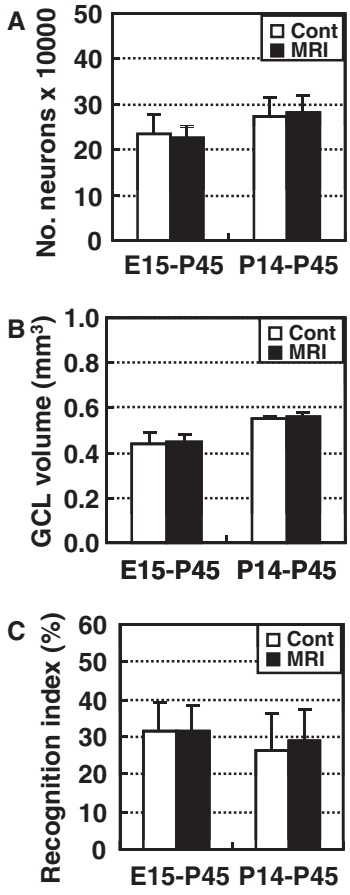

Fig. 4. Neuron numbers in the granule cell layer of the dentate gyrus and object recognition memory. (A) The total number of neurons in the granule cell layer (GCL) of the DG on P45 was counted on DAPI-stained sections using stereological principles. There was no difference between the control and MRI groups. (B) The GCL volumes were calculated on P45 and no differences were observed between the groups. (C) Novel object recognition testing was used to assess memory function. The recognition index, calculated as described in the methods section, was not different between the groups.

GCL 24 h after the last MRI in the P14 rats (Figs. 2A and 3A). The numbers of TUNEL-positive cells in DG was not different between the control and MRI groups (Fig. 3B). Newborn, BrdU-positive cells, did not reveal higher rates of TUNEL staining in the GCL in brains subjected to MRI than in controls (Fig. 3B).

3.3. Neuron numbers in the granule cell layer and recognition memory

The total number of neurons (Fig. 4A) and the volume of the GCL (Fig. 4B) of DG were not different on P45 after MRI, neither in the P14–P45 group, nor in the E15-P45 group. Consequently, the density of neuron in the GCL was not different between any of the group (data not shown). The recognition memory, as judged by the recognition index, was tested in both the E15-P45 and the P14–45 groups and all animals spent more time exploring the novel object. There was no difference between the control and MRI groups (Fig. 4C).

4. Discussion

MRI, unlike X-ray-based imaging techniques, has been considered to be safe also for embryos and young children, but there are no studies addressing possible effects of strong, static magnetic fields on neural stem cell proliferation and differentiation. MRI is widely used as a reliable tool for the diagnosis and follow-up of neonatal brain injuries. Therefore, negative and positive outcomes would be equally important from a clinical point of view. One hypothesis, based on *in vitro* work showing that strong magnetic field exposure of cultured neurons could induce neuronal death [6], was that repeated MRI exposure during brain development would cause cell death in the hippocampus, possibly more pronounced in the prenatal brain. Furthermore, another study found that acute, alternating, low-intensity magnetic field exposure increased apoptosis and necrosis in rat brains and that this could be blocked by pretreating the rats with free radical scavengers [18]. We could, however, not find any negative effects of repeated MRI in the DG of developing brain, neither in late gestation nor in the rapidly growing, postnatal brain.

Magnetic fields can influence biological systems by generating electrical fields, changing diffusion across members, exerting force on charged carriers and magnetic dipoles and catalyzing reactions involving free radicals. It is known that endogenous bioelectromagnetic fields convey morphogenetic information for embryonic development and regeneration [19]. Disruption of endogenous fields was shown to inhibit regeneration. Exogenous, alternating magnetic fields were shown to increase neurogenesis by altering endogenous electric fields [20]. Transcranial magnetic stimulation has been introduced as a therapeutic tool in patients with movement disorders, without apparent side effects [4]. Other studies have reported that neither high (6.3 T) nor low (39 mT) static magnetic fields had any effect on fetal development in mice and rats [21,22], which is in accordance with our results.

Neurogenesis is regulated by physiological and pathological stimuli [9,23,24]. Environmental stimuli may either enhance [23] or suppress [24] stem/progenitor cell proliferation, depending on the relative predominance of enriching or noxious qualities. Given the higher rates of neurogenesis in the prenatal and early postnatal hippocampus, and its role in memory and learning [15], it is reasonable to assume that negative effects on hippocampal neurogenesis early in life may have considerable effects later in life. This has been demonstrated, for example, for early exposure to ionizing radiation, where even a single, moderate dose early postnatally may cause life-long depletion of neurons in the GCL, impaired cognitive function and increased susceptibility to subsequent insults later in life [9,12,13,25–27]. Anesthetics clearly alter the environmental stimuli, including neuronal signaling, and apparently have age-dependent effects on cell proliferation in the hippocampus [28]. In a previous study, we found that isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells and reduced neurogenesis in young, but not adult, rodents [12]. In the present study, animals were anesthetized with isoflurane for the entire duration of the MRI scans. We did not find any differences between the control and MRI groups, but we cannot exclude that MRI had effects that were masked by the effects of isoflurane anesthesia. It was clear, though, that no additive effects of MRI, positive or negative, could be detected. Further studies are needed to confirm these findings in non-anesthetized animals. In summary, this is the first study to our knowledge demonstrating that repeated, prolonged exposure to strong, static magnetic fields during MRI does not affect neurogenesis, cell death or memory function in rats, neither during late gestation nor in the rapidly growing postnatal hippocampus.

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