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Effect of long-term retention of gadolinium on metabolism of deep cerebellar nuclei after repeated injections of gadodiamide in rats

Running title: Gadolinium retention on cerebellar metabolism

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Abstract

Objectives: To determine potential metabolism and histological modifications due to gadolinium retention within deep cerebellar nuclei (DCN) after linear gadolinium based contrast agent injection (gadodiamide) in rats at 1 year after the last injection.

Materials and Methods: Twenty rats received 20 doses of gadodiamide (0.6 mmol of gadolinium per kilogram each) over 5 weeks. They were followed at 1 week (M0), 6 weeks (M1) and 54-55 weeks (M13) post-injections to evaluate hypersignal on unenhanced T1-weighted MRI and metabolic alterations by ^1H MRS. At 1 year post-injections, brains were sampled to determine the localization of gadolinium within cerebellum by laser ablation inductively coupled mass spectroscopy (LA-ICP-MS) and to evaluate morphological changes by semi-quantitative immunofluorescence analysis.

Results: There is a significant increase of the ratio DCN/brainstem for the gadodiamide group at M0 ($+7.2\%$ vs control group $=0.989\pm0.01$), M1 ($+7.6\%$ vs control group $=1.002\pm0.018$) and it lasted up to M13 ($+4.7\%$ vs control group $=0.9862\pm0.008$). No variation among metabolic markers (cellular homeostasis, excitatory neurotransmitter and metabolites specific to a cellular compartment) were detected by ^1H MRS between gadodiamide and saline groups at M0, M1 and M13. At M13, LA-ICP-MS demonstrated that long-term gadolinium retention occurred preferentially in DCN. No histological abnormalities (including analysis of astrocytes, neurons and microglial cells) were found in the rostral part of DCN.

Conclusion: Repeated administration of gadodiamide lead to a retention of gadolinium preferentially within DCN until 1-year post-injections. This retention did not lead to any detectable changes of metabolic biomarkers nor histological alterations.

Key words: gadolinium based contrast agent; cerebellum; deep cerebellar nuclei; gadolinium retention; MRI ; ^1H MRS ; LA-ICP-MS ; histology.

Introduction

Gadolinium is a lanthanide metal with paramagnetic properties extensively used as a contrast agent for magnetic resonance imaging (MRI). For the last 30 years, over 450 million patients have received gadolinium based contrast agent (GBCA) and these have been prescribed in 30-45% of clinical protocols.¹⁻³ However, non-chelated gadolinium (Gd^{3+} form) is highly toxic in biological systems given its similar size as calcium ion (Ca^{2+}).⁴ To avoid this toxicity, Gd^{3+} is chelated with polyaminocarboxylate ligands which can be either macrocyclic or linear. In terms of thermodynamic and kinetic stability, it has been shown that macrocyclic chelates are more stable than their linear counterparts as they demonstrate a lower potential of gadolinium release.⁵

GBCA are extracellular agents which are rapidly cleared from the intravascular space through kidney excretion in patients with normal renal function (90% eliminated during the first 12 hours).⁶ Consequently, GBCA are not supposed to distribute into the central nervous system (CNS) extracellular fluid except if the blood-brain barrier (BBB) is impaired (e.g. tumors, multiple sclerosis, inflammation, irradiation treatment).^{7,8}

Nevertheless, Kanda et al. highlighted the presence of a hypersignal on unenhanced T1-weighted MR images in deep grey matter structures of the brain (deep cerebellar nuclei, DCN and globus pallidus, GP) among patients with normal renal function who had received cumulative doses of GBCA.⁹ Since their seminal article, a positive correlation between this hypersignal in GP and DCN on unenhanced T1-weighted images and the number of repeated intravenous injections of GBCA (up to 6) for MRI exams has been clearly established.⁷⁻¹¹

Moreover, it has been extensively documented that repeated injections of linear GBCAs lead to gadolinium deposition in DCN as opposed to macrocyclic GBCAs.^{10,12-15} Most of the studies were conducted by inductively coupled plasma mass spectrometry (ICP-MS) on post-mortem

74 samples or resections pieces. They have shown the presence of non-negligible quantities of
75 gadolinium in brain tissue of patients.^{16,17} Several histological studies have demonstrated that
76 gadolinium retention occurred without alterations of the BBB.^{16,17}

77 Similar findings were reported in non-clinical rodent models assessed by MRI, ICP-MS and
78 histology.¹⁸ Recently, Robert et al. have demonstrated that gadolinium presence persists at long-
79 term post-injections in brain rats.¹⁹ One year after the last injection of one linear GBCA
80 (gadodiamide), gadolinium has been detected by ICP-MS in cerebellum (2.45 nmol/g), cortical
81 brain (1.23 nmol/g), subcortical brain (1.52 nmol/g) and brainstem (0.74 nmol/g). In the case
82 of macrocyclic GBCA (gadoterate), gadolinium concentrations were in the range of the
83 background noise in these structures (from 0.05 to 0.09 nmol/g). Moreover, gadolinium
84 detected after gadodiamide was found to be no longer in its original form but bound to
85 macromolecules.

86 Several questions are still remaining, including if long-term gadolinium deposition has potential
87 adverse health effects. In our study, we decided to address this question by following rats
88 throughout one year after they received cumulative doses of a linear GBCA (gadodiamide).
89 Thereby, we investigated until one year post-injections if : (i) deposition of GBCA could induce
90 cellular alterations detectable by ¹H magnetic resonance spectroscopy (¹H MRS); (ii) long-term
91 hypersignal on unenhanced T1-weighted MR images is correlated to gadolinium deposition
92 within DCN by laser ablation ICP-MS (LA-ICP-MS) and (iii) histological integrity of cerebellar
93 tissue is compromised.

Materials and Methods

Animals and contrast agent injection

All animal experiments were conducted in accordance with French regulations and in compliance with the European Union Directives 2010/63/EU. Twenty female Sprague-Dawley rats aged 10 weeks (SPF/OFA female rats, Charles River, L'Arbresle, France) were used in this study. The animals were housed under a 12 h light/dark cycle at constant temperature of $23\pm 1^{\circ}\text{C}$ with free access to food and water.

Two experimental groups (10 rats per group) were compared: one group received gadodiamide intravenous injections and the other control group received 0.9% saline intravenous injections. All injections were performed following a defined published protocol under general anesthesia (3%-3.5% isoflurane; IsoFlo, Axience, Pantin, France).²⁰ The gadodiamide (Omniscan®, 0.5 M, GE Healthcare, Chalfont-St-Giles, United Kingdom) group received 20 intravenous injections of 0.6 mmol of gadolinium per kilogram (1.2 mL/kg) over a period of 5 weeks (4 daily and consecutive injections per week). The daily dose of 0.6 mmol gadolinium per kilogram is equivalent to the usual human dose of GBCAs (0.1 mmol of gadolinium per kilogram or 0.2 mL/kg), after adjustment for body surface area as recommended by the Food and Drug Administration²¹. A clinical follow up of animals was regularly performed.

MR experiments

During the MR experiments, the animals were positioned in a custom-built cradle to immobilize the head. They were anesthetized during the MR experiment with 1.5%-2% isoflurane and a mixture of $\text{O}_2/\text{N}_2\text{O}$ (1:1) with an output of 0.5 L/min. Respiration was monitored during the whole experiment using an air pillow and body temperature was maintained constant at 37°C by a warm-water circulation system. MR acquisitions were performed on a 7T horizontal ultra-shielded superconducting magnet dedicated to small animal imaging (70/16 Bruker

Pharmascan, Wissembourg, France) and equipped with a 230 mT/m gradient set. A Bruker 38 mm inner diameter birdcage coil was used for both ^1H transmission and reception.

All rats underwent the same protocol (**figure 1**). MR experiments were performed at M0 (one week after the last injection; 0 month of washout period), M1 (six weeks after the last injection; 1 month of washout period) and M13 (54-55 weeks after the last injection; 13 months of washout period).

An unenhanced T1-weighted gradient echo sequence (intra-gate fast low angle shot; Ig-FLASH) was used with the following parameters: bandwidth= 75 kHz; TR/TE= 129 ms/2.3 ms; 6 averages; in plane resolution= $78 \times 78 \mu\text{m}^2/\text{pixel}$; slice thickness= $570 \mu\text{m}$; 15 slices; respiration frequency for reconstruction= 40 breaths per minute; acquisition time= 6 minutes 38 seconds.

PRESS (point resolved spectroscopy) sequence was acquired with the following parameters: voxel size= $3 \times 2 \times 1.5 \text{ mm}^3$; TR/TE=2775 ms/16 ms; spectral bandwidth= 4006.41 Hz (13.34 ppm); 2048 points; 970 averages; acquisition time= 44 min 52 seconds. T1-weighted images were used to place the voxel in the left DCN with an extended care to avoid brainstem and subcutaneous lipids. A VAPOR module (variable pulse power and optimized relaxation delays) for water suppression was achieved to assess only cerebellar metabolites. An OVS module (outer volume suppression) was used to eliminate signal outside the voxel. Localized first and second order shim terms were adjusted within the voxel.

Quantification of T1-weighted MR Image

Quantification was performed in a blinded manner. Two regions of interest (ROI) were positioned in the most visible right or left deep cerebellar nuclei (DCN), and in the brainstem (reference). Signal intensity was calculated as the ratio between the signal of the more visible of the two DCN signal and the brainstem signal (taken as a reference).

Spectral analysis and quantification

Spectra were analyzed on CSIapo software developed by Yann Le Fur (CRMBM, Marseille, France) and Helene Ratiney (CREATIS – INSA, Lyon, France) which is an interface for quantification algorithm QUEST (quantitation based on quantum estimation).²² QUEST is a linear combination model decomposing free induction decay (FID) signal in temporal domain. QUEST uses a database to fit a weighted combination of metabolite signals and then to obtain a quantitation in arbitrary unit (A.U). This database is simulated through GAVA (Gamma Visual Analysis) which is used for modelling metabolites signals including their characteristics (chemical shift, amplitude and phase) and also experimental parameters (magnetic field and echo time).²³

Cerebral metabolites quantified in DCN are the following: creatine and phosphocreatine (Cr+PCr); N-Acetyl Aspartate (NAA); choline, phosphocholine and glycérophosphocholine (tCho); glutamate (Glu); glutamate+glutamine (Glx); Taurine (Tau) and myo-Inositol (mI) (**figure 2**).

Sample collection

Following MR experiments, animals were sacrificed under general anesthesia and analgesia (isoflurane, 5%). The sacrifice consisted in exsanguination and perfusion of the blood system with saline. Immediately after euthanasia of rats, the skull was opened and the brain was taken off. The cerebellum was sampled and placed in OCT medium and then immediately cooled down in isopentane (-60°C) until complete freezing. Frozen specimens were stored at -80°C. The cerebellum was cut at -20°C in serial 7 µm-thick sections at the level of deep nuclei, according to the atlas of Paxinos and Watson²⁴. The appropriate level of section were validated with cresyl violet staining. The rostral sections of the DCN were used for immunostaining protocols and the caudal portions were sampled for LA-ICP-MS experiments.

Histology

Slides at the level of DCN were used for the immunostaining protocol (n=6/group) in purpose to detect 3 targets of interest: GFAP (glial fibrillary acidic protein, astrocytes maker), synaptophysin (SYP; neurons) and CD68 (microglial cells). Alexa 488 was chosen as the fluorescent dye to detect the targeted molecule. The primary antibodies and corresponding secondary antibody were selected as shown in **table 1**. The immunostaining protocol was performed for each marker as detailed in **table 2**. At the end of the immunostaining, slides were cover slipped with a medium containing DAPI (4',6-diamidino-2-phenylindole) for nuclear counterstain.

After immunostaining, images of DCN slides were acquired by epifluorescence microscopy using a FITC filter cube with similar image acquisition parameters for all slides (1280 x 1024 pixels, exposure time 300 ms, gain 1, medium contrast). Semi-quantitative analysis of GFAP, SYP and CD68 signals were performed by evaluating the signal distribution and its prevalence on a scale from 0 (no signal) to 3 (high density of signal) within the deep cerebellar nuclei area.

LA-ICP-MS

For quantitative LA-ICP-MS analysis, matrix matched standards based on gelatin were used for external calibration. Seven gadolinium standards with concentrations ranging from 0 to 50 µg/g were prepared by diluting Gd ICP Standard (TraceCERT®, 1000 mg/L Gd, Sigma-Aldrich, MO, USA) and spiking 10% w/w gelatin (Grüssing GmbH, Filsum, Germany). These standards were heated up to 40°C and homogenized. For quantification of iron, copper and zinc, multielement standards with concentrations ranging from 0 to 100 µg/g per element were prepared in the same way as the Gd standards. Concentrations of the prepared standards were verified by bulk analysis with ICP-MS (iCap TQ, Thermo Fisher, Bremen, Germany) after acidic digestions.

190 Gelatin-based standards and the samples (n=6/group) were sliced into 10 µm thick sections with
191 a cryotome (CryoStar NX70, Thermo Fisher, Bremen, Germany) and mounted onto glass slides.
192 LA-ICP-MS analysis was performed with a laser ablation system (LSX G2+, Cetac
193 Technologies, Omaha, USA) equipped with 213 nm Nd:YAG laser, a two-volume HelEX
194 ablation cell and connective tygon tubings, connected to a ICP-MS (iCap TQ, Thermo Fisher,
195 Bremen, Germany) equipped with Ni sampler and skimmer cone and a quartz injector with an
196 inner diameter of 2.5 mm.
197 Laser energy was adjusted to allow for complete ablation of the samples. For calibration, ten
198 parallel lines and for the samples, the whole brain section was ablated linewise with a laser spot
199 size of 25 µm and a scan speed of 75 µm/s.

200 [Statistical analysis](#)

201 Data are given as mean ± SEM. Statistical analyses were performed using Prism 5.1 (GraphPad,
202 La Jolla, CA, USA). Non-parametric t test Mann-Whitney was used on weight, MRI and ¹H
203 MRS data for comparison between saline and gadodiamide groups. A p<0.05 was considered
204 statistically significant.

Results

Sanitary following of animals

Four rats of the gadodiamide group died during the study period: 1 unexplained death (at M0), 3 ethically sacrificed (one at M0 and 2 at M1) because signs of pain induced by visible ulcerative and squamous skin lesions. One rat in the saline group died at M0 during the anesthesia.

Qualitative analysis of T1-weighted MR images

Figure 3 shows examples of typical non enhanced T1-weighted images obtained for both groups (saline and gadodiamide) 1 week (M0), 6 weeks (M1) and 54-55 weeks (M13) after the end of the injections.

Specific DCN signal enhancement (arrows) is present for all rats of the gadodiamide group. The hyperintensity was present on the images as early as the week following the end of the injections and persisted over time even 1 year after the end of the injections.

Quantitative analysis of signal intensity in T1-weighted images

The ratio between the signals of the DCN and the brainstem for M0, M1 and M13 are presented on **figure 4**. There is a significant increase of the ratio DCN/brainstem for the gadodiamide group at M0 (1 week after the end of the injections) and M1 (6 weeks after the end of the injections). This increase in DCN signal intensity lasted up to 1 year after the end of the injections.

At M0, the ratio for DCN signal intensity to brainstem increased by 7.2% in gadodiamide group (1.060 ± 0.010) compared to saline group (0.989 ± 0.01). At M1, the ratio for DCN signal intensity to brainstem was elevated of 7.6% for gadodiamide group (1.078 ± 0.014) vs saline group (1.002 ± 0.018). At M13, the ratio for DCN signal intensity to brainstem was greater of 4.7% for gadodiamide group (1.033 ± 0.011) in comparison of saline group (0.9862 ± 0.008).

MR Spectroscopy

Figure 5 represents the concentration of metabolites measured in the DCN at M0 (1 week post-injections), M1 (6 weeks post-injections) and M13 (54-55 weeks post-injections). MRS allows to detect three categories of metabolites: (i) cellular homeostasis (creatine, choline, taurine); (ii) excitatory neurotransmitter (glutamate) and (iii) metabolites specific to a cellular compartment (NAA for neurons and myo-Inositol for glial cells). No variation among these markers was detected between gadodiamide and saline groups at M0, M1 and M13.

LA-ICP-MS

The element distribution for Gd, P, Fe, Cu and Zn in rat brain observed after repeated injection of either gadodiamide (left side) or saline solution (right side) are shown in **figure 6**. Corresponding microscopic images of the analysed thin sections are also shown (a,b) for comparison. Concentrations up to 64 nmol/g, in a few cases even higher, were found in the brain of rats treated with gadodiamide. Highest concentrations were found in the region of DCN, with average concentrations ranging from 25 to 35 nmol/g in the three substructures. For the rat brain injected with saline solution, no Gd could be detected.

No clear correlation between gadolinium and phosphorous, copper or zinc can be observed, but the highest Gd concentrations are found in the region with highest iron concentrations. The average iron concentrations are slightly lower (497 nmol/g) in the DCN of the gadodiamide sample compared to the untreated animal (665 nmol/g).

Immunofluorescent analysis

Semi-quantitative analysis of cerebellar cell populations (astrocytes, neurons and glial cells) were performed at 1-year post-injections in gadodiamide and saline groups. GFAP is a marker for astrocyte cells (density and thickness of astrocyte processes) and allowed to determine their distribution (perineuronal and perivascular). Synaptophysin is a neuronal

253 marker which provide a morphological information on perikaryon. CD68 is a marker for
254 microglial cells which indicate their distribution (perivascular activated microglial) and their
255 morphology (diffuse neuropil) (**figure 7a**).

256 No morphological changes were observed between gadodiamide and saline groups based on
257 the evaluation of immunofluorescent analysis of various cells populations in the deep cerebellar
258 nuclei area (**figure 7b**).

Discussion

The main goal of our study was to evaluate the long-term retention of a linear gadolinium based contrast agent (GBCA; gadodiamide) within the cerebellum, and more specifically in the deep cerebellar nuclei (DCN) after 1-year post-injections. We used both *in vivo* MR techniques (MRI and ^1H MRS) as well as quantitative chemical (LA-ICP-MS) and histological approaches.

In our experimental design, we used a rodent model receiving cumulative doses of a linear GBCA (12 mmol of gadolinium per kilogram) which was previously demonstrated to lead to a cerebellar gadolinium accumulation at 5 weeks post-injections and also at 1 year post-injections.^{19,20,25} Animal welfare was followed throughout our longitudinal MR study to monitor any physiological changes induced by repeated injections of gadodiamide.

This clinical monitoring allowed us to notice the apparition of skin lesions among our animals (3 out of 10 rats) between 1 and 5 weeks post-injections. Lohrke et al. have reported similar observations of skin injuries among 4 out of 10 rats exposed to cumulative doses of gadodiamide (50 mmol of gadolinium per kilogram; 4 times higher than in our protocol).²⁶ In their protocol, a high concentration of gadolinium was quantified in the skin after gadodiamide exposure (1472 nmol/g) and not in the case of others GBCAs (gadopentetate dimeglumine, gadobutrol and gadoteridol). Moreover, several studies have shown that there is an accumulation of gadolinium in tissues such as bone and skin both in humans and rats with normal renal function.^{27–29}

Throughout our longitudinal study, we observed a T1 hypersignal in cerebellum on unenhanced MR images at 1 and 5-weeks post-injections which was persistent until 13 months post-injections. This observation is in agreement with a previous report on the same rat model at 1-year post-injections, gadolinium concentration was quantified up to 2.45 nmol/g within cerebellum.¹⁹ Our results obtained by LA-ICP-MS demonstrated that long-term gadolinium

retention occurred preferentially in DCN. This result is consistent with previous studies that showed preferential gadolinium accumulation in this cerebellar structure at an earlier point (8 weeks post-injections).²⁶ Given the high concentration of metals within DCN, the most likely hypothesis would be that GBCA dechelated through a transmetalation of gadolinium to iron, copper or zinc. The limit of LA-ICP-MS method lies in its incapacity to identify whether gadolinium is present under its chelated or dechelated form.

Despite gadolinium retention within DCN, we did not observed any morphological modification of various cellular populations (neurons, astrocytes and microglia) at 1-year post-injections. Previous studies at earlier time points (8 and 20 weeks post-injections) are concordant with our results.^{26,30} So far, histological studies only explored morphological features. Furthers studies should focus on inflammatory markers to determine whether gadolinium retention in DCN could induce a local inflammation.

Our main goal was to identify whether repeated injections of gadodiamide could lead to metabolic alterations at short (1 and 6 weeks post-injections) and long-term (1-year post-injections) using ¹H MRS. To date, few data are available for cerebellar metabolism following repeated injections of linear GBCA. Our main observation was that long-term retention of gadodiamide did not induce any detectable metabolic changes in the cerebellum after 1-year post-injections. In accordance with literature, ¹⁸F-fluorodeoxyglucose positron emission tomography (¹⁸F-FDG-PET) imaging of rats who had received a cumulative dose of gadodiamide (13.2 mmol/kg over 7 weeks) did not reveal any abnormal glucose uptake in the DCN, and thus even with chronic cerebral hypoperfusion (model of aging and neurodegenerative diseases).³¹ However, a ¹⁸F-FDG-PET study had shown a lower maximum standardized uptake value (SUVmax) in DCN of patients who had received repeated injections of GBCAs (3 to 6 injections without distinction between macrocyclic and linear forms)

307 compared to those who had not received, which might indicate a decrease of glucose
308 metabolism.³²

309 Nevertheless, it should be taken under consideration that ¹H MRS may not be sensitive enough
310 to detect any subtle modifications of cerebellar metabolism. Indeed, one technical limit of ¹H
311 MRS is its inability to detect molecules below mM range. It also appears that metabolites
312 involved in cellular homeostasis and excitatory neurotransmitter (glutamate) which are
313 detectable by ¹H MRS may not be a target of dechelated gadolinium. Another methodological
314 limit of *in vivo* ¹H MRS is that a technique sensitive only to molecules with a high degree of
315 rotational molecular motion.³³ Moreover, it has been recently reported that gadolinium linked
316 preferentially to macromolecules (larger to 66.5 kDa in insoluble fraction).¹⁹ In the case of
317 gadolinium bounded to macromolecules such as proteins or lipids and especially within the
318 membrane bilayer, any alteration could not be detectable by ¹H MRS.

319 In our study, we studied only one representative of linear GBCAs which is neutral in terms of
320 charge. Indeed, linear ionic GBCAs such as gadobenate dimeglumine and gadopentetate
321 dimeglumine induce also gadolinium retention in the cerebellum of patients.^{11,15,34} Furthermore,
322 linear ionic GBCA have been previously shown to decrease the area under the peak of choline
323 in breast and cerebral tumors.^{35–37} The peak of choline is widely used in diagnostic as an index
324 for cellular density of the tumor and the degree of tumor infiltration into brain tissue.³⁸ It will
325 be highly interesting to investigate the impact of linear ionic GBCAs on ¹H MRS spectra in
326 brain. In this purpose, further studies must be conducted to evaluate if they can alter choline
327 peak signal and thereby diagnostic.

328 To conclude, we demonstrate that cumulative administration of a linear and nonionic GBCA
329 (gadodiamide) induces a retention of gadolinium within DCN at 1-year post-injections and thus
330 without altering metabolic biomarkers of cellular homeostasis and an excitatory
331 neurotransmitter (glutamate). Although, histological analysis did not show any alterations of

332 DCN, further studies are required to elucidate the putative clinical long-term consequences of
333 gadolinium retention (e.g. inflammatory markers) as well as the mechanism underlying such a
334 retention.

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432

Tables

Table 1: Primary antibodies and corresponding antibodies for immunostaining protocol

Primary antibody	Clone	Secondary antibody
Mouse anti-CD68 IgG1	ED1	Anti-mouse IgG1 A488
Rabbit anti-SYP	YE269	Anti-rabbit IgG A488
Rabbit anti-GFAP	polyclonal	Anti-rabbit IgG A488

Table 2: Steps for immunostaining protocol

Step	Reagent	Condition
Fixation of slides	Formalin 4%	10 min at room temperature
Rinsing	PBS1x	3 x 5 min
Serum blocking	BSA 3% / Goat serum 10% in PBS1x	1h at room temperature
Primary antibody incubation	Anti-GFAP 1/1000 ^e Anti-CD68 1/200 ^e Anti-SYP 1/600 ^e	1h at room temperature 1h at room temperature 1h at room temperature
Rinsing	PBS 1x	3 x 5 min
Secondary antibody	Anti-IgG A488 1/500 ^e	1h at room temperature
Rinsing	PBS1x	3 x 5 min

Figure legends

Figure 1: MRI, ¹H MRS and injection scheme. MR experiments are performed at M0 (Week 6, one week after the last injection; 0 month of washout period), M1 (Week 11, six weeks after the last injection; 1 month of washout period) and M13 (Week 59, 53 weeks after the last injection; 13 months of washout period). After MRI, animals are sacrificed and samples are used for histological and LA-ICP-MS analysis.

Figure 2: (A) Voxel position in the left dentate cerebellar nuclei (DCN) on T1-weighted images; (B) Typical MRS spectra obtained from gadodiamide rat at 1-week post-injection. The peaks assignments are as follows: macromolecules (MM); creatine and phosphocreatine (Cr+PCr); N-Acetyl Aspartate (NAA); choline, phosphocholine and glycerophosphocholine (tCho); glutamate (Glu); glutamate+glutamine (Glx); taurine (Tau); myo-Inositol (mI).

Figure 3: T1-weighted Ig-FLASH images at 7T of saline and gadodiamide rats at M0, M1 and M13. MR images of Rat n°10 (saline group) and Rat n°18 (gadodiamide group) are qualitatively followed at the three time points.

Figure 4: Delay Effect - Quantitative analysis of enhancement at M0, M1 and M13. Signals ratio DCN/Brainstem were measured for saline and gadodiamide groups (values are mean $\pm$ SEM). *** $p < 0.001$; ** $p < 0.01$ and * $p < 0.05$

Figure 5: MR Spectroscopy quantitation obtained with QUEST algorithm at delay M0, M1 and M13. Measured metabolites concentration are as follows: N-Acetyl Aspartate (NAA); creatine and phosphocreatine (Cr+PCr); choline, phosphocholine and glycerophosphocholine (tCho); taurine (Tau); myo-Inositol (mI) and glutamate (Glu). Values are mean $\pm$ SEM.

461 **Figure 6: LA-ICP-MS analysis of rat brains after repeated injections of gadodiamide (left)**
462 **and saline solution (right).** Phase contrast microscopic images (a,b) of the analyzed thin
463 sections with quantified distribution of Gd (c,d), Fe (g,h), Cu (i,j) and Zn (k,l) as well as the
464 qualitative distribution of P (e,f).

465 **Figure 7: (a) Representative images of GFAP and CD68 immunostaining within deep**
466 **cerebellar nuclei of gadodiamide rat; (b) Semi-quantitative immunofluorescence analysis**
467 **of GFAP, synaptophysin (SYP) and CD68 signals.** Scale were evaluated from 0 (no signal)
468 to 3 (high density of signal) within the deep cerebellar nuclei area. Values are mean $\pm$ SEM.