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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**

Dounia El Hamrani, PhD<sup>1</sup>; Véronique Vives, PhD<sup>2</sup>; Rebecca Buchholz, MSc<sup>3</sup>, William Mème, PhD<sup>1</sup>; Cecile Factor, PhD<sup>2</sup>; Stefanie Fingerhut, PhD<sup>3</sup>; Michael Sperling, PhD<sup>3</sup>; Uwe Karst, PhD<sup>3</sup>; Philippe Robert, PhD<sup>2</sup>, Sandra Mème, PhD<sup>1</sup>

<sup>1</sup> Center of Molecular Biophysics, CNRS UPR4301, Rue Charles Sadron, CS 80054, 45071, Orléans Cedex 2, France

<sup>2</sup> Department of Research and Innovation, Imaging and Biological Research Division, Guerbet Group, BP57400, 95943 Roissy CDG, France

<sup>3</sup> Institute of Inorganic and Analytical Chemistry, University of Münster, Corrensstraße 30, 48149, Münster, Germany

**Correspondence to:** Sandra Mème, Center of Molecular Biophysics, CNRS UPR4301, Rue Charles Sadron, CS 80054, 45071, Orléans Cedex 2, France

Phone: +33 (0)2 38 25 51 07; Fax: +33 (0)2 38 63 15 17; Email: [sandra.meme@cnrs.fr](mailto:sandra.meme@cnrs.fr)

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## Abstract

26

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

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

37 **Results:** There is a significant increase of the ratio DCN/brainstem for the gadodiamide group  
38 at M0 (+7.2% vs control group=0.989±0.01), M1 (+7.6% vs control group=1.002±0.018) and  
39 it lasted up to M13 (+4.7% vs control group=0.9862±0.008). No variation among metabolic  
40 markers (cellular homeostasis, excitatory neurotransmitter and metabolites specific to a cellular  
41 compartment) were detected by <sup>1</sup>H MRS between gadodiamide and saline groups at M0, M1  
42 and M13. At M13, LA-ICP-MS demonstrated that long-term gadolinium retention occurred  
43 preferentially in DCN. No histological abnormalities (including analysis of astrocytes, neurons  
44 and microglial cells) were found in the rostral part of DCN.

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

48 **Key words:** gadolinium based contrast agent; cerebellum; deep cerebellar nuclei; gadolinium  
49 retention; MRI ; <sup>1</sup>H MRS ; LA-ICP-MS ; histology.

## 50 Introduction

51 Gadolinium is a lanthanide metal with paramagnetic properties extensively used as a contrast  
52 agent for magnetic resonance imaging (MRI). For the last 30 years, over 450 million patients  
53 have received gadolinium based contrast agent (GBCA) and these have been prescribed in 30-  
54 45% of clinical protocols.<sup>1-3</sup> However, non-chelated gadolinium ( $Gd^{3+}$  form) is highly toxic in  
55 biological systems given its similar size as calcium ion ( $Ca^{2+}$ ).<sup>4</sup> To avoid this toxicity,  $Gd^{3+}$  is  
56 chelated with polyaminocarboxylate ligands which can be either macrocyclic or linear. In terms  
57 of thermodynamic and kinetic stability, it has been shown that macrocyclic chelates are more  
58 stable than their linear counterparts as they demonstrate a lower potential of gadolinium  
59 release.<sup>5</sup>

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

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

71 Moreover, it has been extensively documented that repeated injections of linear GBCAs lead to  
72 gadolinium deposition in DCN as opposed to macrocyclic GBCAs.<sup>10,12-15</sup> Most of the studies  
73 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.<sup>16,17</sup> Several histological studies have demonstrated that  
76 gadolinium retention occurred without alterations of the BBB.<sup>16,17</sup>

77 Similar findings were reported in non-clinical rodent models assessed by MRI, ICP-MS and  
78 histology.<sup>18</sup> Recently, Robert et al. have demonstrated that gadolinium presence persists at long-  
79 term post-injections in brain rats.<sup>19</sup> 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 <sup>1</sup>H magnetic resonance spectroscopy (<sup>1</sup>H 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.

## 94 Materials and Methods

### 95 Animals and contrast agent injection

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

101 Two experimental groups (10 rats per group) were compared: one group received gadodiamide  
102 intravenous injections and the other control group received 0.9% saline intravenous injections.  
103 All injections were performed following a defined published protocol under general anesthesia  
104 (3%-3.5% isoflurane; IsoFlo, Axience, Pantin, France).<sup>20</sup> The gadodiamide (Omniscan®, 0.5  
105 M, GE Healthcare, Chalfont-St-Giles, United Kingdom) group received 20 intravenous  
106 injections of 0.6 mmol of gadolinium per kilogram (1.2 mL/kg) over a period of 5 weeks (4  
107 daily and consecutive injections per week). The daily dose of 0.6 mmol gadolinium per  
108 kilogram is equivalent to the usual human dose of GBCAs (0.1 mmol of gadolinium per  
109 kilogram or 0.2 mL/kg), after adjustment for body surface area as recommended by the Food  
110 and Drug Administration<sup>21</sup>. A clinical follow up of animals was regularly performed.

### 111 MR experiments

112 During the MR experiments, the animals were positioned in a custom-built cradle to immobilize  
113 the head. They were anesthetized during the MR experiment with 1.5%-2% isoflurane and a  
114 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  
115 whole experiment using an air pillow and body temperature was maintained constant at  $37^{\circ}\text{C}$   
116 by a warm-water circulation system. MR acquisitions were performed on a 7T horizontal ultra-  
117 shielded superconducting magnet dedicated to small animal imaging (70/16 Bruker

118 Pharmascan, Wissembourg, France) and equipped with a 230 mT/m gradient set. A Bruker  
119 38 mm inner diameter birdcage coil was used for both <sup>1</sup>H transmission and reception.

120 All rats underwent the same protocol (**figure 1**). MR experiments were performed at M0 (one  
121 week after the last injection; 0 month of washout period), M1 (six weeks after the last injection;  
122 1 month of washout period) and M13 (54-55 weeks after the last injection; 13 months of  
123 washout period).

124 An unenhanced T1-weighted gradient echo sequence (intra-gate fast low angle shot; Ig-  
125 FLASH) was used with the following parameters: bandwidth= 75 kHz; TR/TE= 129 ms/2.3  
126 ms; 6 averages; in plane resolution= 78 x 78 μm<sup>2</sup>/pixel; slice thickness= 570 μm; 15 slices;  
127 respiration frequency for reconstruction= 40 breaths per minute; acquisition time= 6 minutes  
128 38 seconds.

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

### 137 [Quantification of T1-weighted MR Image](#)

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

## 142 Spectral analysis and quantification

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

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

## 156 Sample collection

157 Following MR experiments, animals were sacrificed under general anesthesia and analgesia  
158 (isoflurane, 5%). The sacrifice consisted in exsanguination and perfusion of the blood system  
159 with saline. Immediately after euthanasia of rats, the skull was opened and the brain was taken  
160 off. The cerebellum was sampled and placed in OCT medium and then immediately cooled  
161 down in isopentane (-60°C) until complete freezing. Frozen specimens were stored at -80°C.  
162 The cerebellum was cut at -20°C in serial 7 µm-thick sections at the level of deep nuclei,  
163 according to the atlas of Paxinos and Watson<sup>24</sup>. The appropriate level of section were validated  
164 with cresyl violet staining. The rostral sections of the DCN were used for immunostaining  
165 protocols and the caudal portions were sampled for LA-ICP-MS experiments.



## 166 Histology

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

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

## 180 LA-ICP-MS

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

190 Gelatin-based standards and the samples (n=6/group) were sliced into 10  $\mu$ m 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 HeEX  
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  $\mu$ m and a scan speed of 75  $\mu$ m/s.

#### 200 [Statistical analysis](#)

201 Data are given as mean  $\pm$  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  $^1$ H  
203 MRS data for comparison between saline and gadodiamide groups. A  $p < 0.05$  was considered  
204 statistically significant.

## 205 Results

### 206 Sanitary following of animals

207 Four rats of the gadodiamide group died during the study period: 1 unexplained death (at M0),  
208 3 ethically sacrificed (one at M0 and 2 at M1) because signs of pain induced by visible  
209 ulcerative and squamous skin lesions. One rat in the saline group died at M0 during the  
210 anesthesia.

### 211 Qualitative analysis of T1-weighted MR images

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

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

### 218 Quantitative analysis of signal intensity in T1-weighted images

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

224 At M0, the ratio for DCN signal intensity to brainstem increased by 7.2% in gadodiamide group  
225 ( $1.060 \pm 0.010$ ) compared to saline group ( $0.989 \pm 0.01$ ). At M1, the ratio for DCN signal  
226 intensity to brainstem was elevated of 7.6% for gadodiamide group ( $1.078 \pm 0.014$ ) vs saline  
227 group ( $1.002 \pm 0.018$ ). At M13, the ratio for DCN signal intensity to brainstem was greater of  
228 4.7% for gadodiamide group ( $1.033 \pm 0.011$ ) in comparison of saline group ( $0.9862 \pm 0.008$ ).

## 229 MR Spectroscopy

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

## 236 LA-ICP-MS

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

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

## 248 Immunofluorescent analysis

249 Semi-quantitative analysis of cerebellar cell populations (astrocytes, neurons and glial cells)  
250 were performed at 1-year post-injections in gadodiamide and saline groups.

251 GFAP is a marker for astrocyte cells (density and thickness of astrocyte processes) and allowed  
252 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**).

## 259 Discussion

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

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

269 This clinical monitoring allowed us to notice the apparition of skin lesions among our animals  
270 (3 out of 10 rats) between 1 and 5 weeks post-injections. Lohrke et al. have reported similar  
271 observations of skin injuries among 4 out of 10 rats exposed to cumulative doses of  
272 gadodiamide (50 mmol of gadolinium per kilogram; 4 times higher than in our protocol).<sup>26</sup> In  
273 their protocol, a high concentration of gadolinium was quantified in the skin after gadodiamide  
274 exposure (1472 nmol/g) and not in the case of others GBCAs (gadopentetate dimeglumine,  
275 gadobutrol and gadoteridol). Moreover, several studies have shown that there is an  
276 accumulation of gadolinium in tissues such as bone and skin both in humans and rats with  
277 normal renal function.<sup>27-29</sup>

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

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

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

295 Our main goal was to identify whether repeated injections of gadodiamide could lead to  
296 metabolic alterations at short (1 and 6 weeks post-injections) and long-term (1-year post-  
297 injections) using <sup>1</sup>H MRS. To date, few data are available for cerebellar metabolism following  
298 repeated injections of linear GCBA. Our main observation was that long-term retention of  
299 gadodiamide did not induce any detectable metabolic changes in the cerebellum after 1-year  
300 post-injections. In accordance with literature, <sup>18</sup>F-fluorodeoxyglucose positron emission  
301 tomography (<sup>18</sup>F-FDG-PET) imaging of rats who had received a cumulative dose of  
302 gadodiamide (13.2 mmol/kg over 7 weeks) did not reveal any abnormal glucose uptake in the  
303 DCN, and thus even with chronic cerebral hypoperfusion (model of aging and  
304 neurodegenerative diseases).<sup>31</sup> However, a <sup>18</sup>F-FDG-PET study had shown a lower maximum  
305 standardized uptake value (SUVmax) in DCN of patients who had received repeated injections  
306 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.<sup>32</sup>

309 Nevertheless, it should be taken under consideration that <sup>1</sup>H MRS may not be sensitive enough  
310 to detect any subtle modifications of cerebellar metabolism. Indeed, one technical limit of <sup>1</sup>H  
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 <sup>1</sup>H MRS may not be a target of dechelated gadolinium. Another methodological  
314 limit of *in vivo* <sup>1</sup>H MRS is that a technique sensitive only to molecules with a high degree of  
315 rotational molecular motion.<sup>33</sup> Moreover, it has been recently reported that gadolinium linked  
316 preferentially to macromolecules (larger to 66.5 kDa in insoluble fraction).<sup>19</sup> 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 <sup>1</sup>H 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.<sup>11,15,34</sup> Furthermore,  
322 linear ionic GBCA have been previously shown to decrease the area under the peak of choline  
323 in breast and cerebral tumors.<sup>35-37</sup> 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.<sup>38</sup> It will  
325 be highly interesting to investigate the impact of linear ionic GBCAs on <sup>1</sup>H 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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## Tables

434 **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

435

436 **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 <sup>e</sup> Anti-CD68 1/200 <sup>e</sup> Anti-SYP 1/600 <sup>e</sup>	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 <sup>e</sup>	1h at room temperature
Rinsing	PBS1x	3 x 5 min

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438

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## Figure legends

440 **Figure 1: MRI, <sup>1</sup>H MRS and injection scheme.** MR experiments are performed at M0 (Week  
441 6, one week after the last injection; 0 month of washout period), M1 (Week 11, six weeks after  
442 the last injection; 1 month of washout period) and M13 (Week 59, 53 weeks after the last  
443 injection; 13 months of washout period). After MRI, animals are sacrificed and samples are  
444 used for histological and LA-ICP-MS analysis.

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

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

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

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

460

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.