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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

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 ^1H 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 ^1H 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 ; ^1H 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.¹⁻³ However, non-chelated gadolinium (Gd^{3+} form) is highly toxic in
55 biological systems given its similar size as calcium ion (Ca^{2+}).⁴ 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.⁵

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).⁶ 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).^{7,8}

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.⁹ 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.⁷⁻¹¹

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.^{10,12-15} 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.^{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.

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).²⁰ 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 Administration²¹. 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°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 ¹H 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 μm²/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 mm³; 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).²² 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).²³

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 Watson²⁴. 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 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 HeIEX
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.

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 ± 0.010) compared to saline group (0.989 ± 0.01). At M1, the ratio for DCN signal
226 intensity to brainstem was elevated of 7.6% for gadodiamide group (1.078 ± 0.014) vs saline
227 group (1.002 ± 0.018). At M13, the ratio for DCN signal intensity to brainstem was greater of
228 4.7% for gadodiamide group (1.033 ± 0.011) in comparison of saline group (0.9862 ± 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 ¹H 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.^{19,20,25} 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).²⁶ 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.²⁷⁻²⁹

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.¹⁹ 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).²⁶ 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.^{26,30} 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 ¹H 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, ¹⁸F-fluorodeoxyglucose positron emission
301 tomography (¹⁸F-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).³¹ However, a ¹⁸F-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.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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433

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 ^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

437

438

439

Figure legends

440 **Figure 1: MRI, ¹H 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.