AMERICAN UNIVERSITY OF BEIRUT

DOES GADOLINIUM DEPOSITION IN THE BRAIN AFFECT HIPPOCAMPAL NEUROGENESIS?

by
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This work is dedicated to my country, the Kingdom of Saudi Arabia that I carry in my heart and soul. It is also dedicated to my family, especially my mother and sister Insaf. Thank you for your infinite continuous support, I am grateful to have you. Finally and most importantly, it is dedicated to patients with neurological disorders. I hope to be able to serve you throughout my scientific journey…

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AN ABSTRACT OF THE THESIS OF

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Title: Does Gadolinium Deposition in the Brain affect Hippocampal Neurogenesis?

Background and Aims: Gadolinium-based contrast agents (GBCAs) are the only FDA approved agents that are used worldwide to enhance the visualization of internal body organs and tissues on MRI scans. Recent postmortem studies have shown that the exposure to linear and macrocyclic GBCAs result in Gadolinium (Gd) metal deposition in the brain and other organs. While the clinical significance of such metal deposition remains unsettled, it raises important questions concerning its long-term effects on learning and memory in developing brains undergoing multiple MRI scans. The purpose of this study is to investigate whether repeated exposure to linear and macrocyclic GBCAs at young age have an impact on the stem cell niche in the hippocampus, or affect the working memory performance. It also aims at investigating if exposure to GBCAs leads to Gd deposits in the spinal cord and peripheral nerves.

Methods: Young male Sprague Dawley rats (140-150 g) were given serial daily intraperitoneal injections of two types of GBCAs: Gadoterate meglumine (Dotarem; macrocyclic GBCA) and Gadodiamide (Omniscan; Linear GBCA) at a dose of 2.5 mmol/kg for a period of 20 days. A control group received Saline injections. Along with GBCAs, animals received Bromodeoxyuridine injections every three days (total dose = 300 mg/kg; *ip*) to label newly formed cells in the brain. In order to assess the total number of proliferating cells in the dentate-gyrus of the hippocampus, one set of animals was sacrificed 48 hours after the last BrdU injection by cardiac perfusion. Furthermore, to determine the number of newly maturing neurons in the dentate gyrus, another set was sacrificed 29 days after the last BrdU injection. Hippocampal tissues were stained for $BrdU^+$ and $NeuN^+$ cells and quantified using confocal microscopy. The spontaneous alternation T-maze test was performed to assess the spatial working memory function at day 10, at day 20, and one month after the last GBCA exposure. Inductively Coupled Plasma Mass-Spectrometry (ICP-MS) analysis was used to detect and quantify Gd metal in the brains, spinal cords, and peripheral nerves (Sciatic and Trigeminal nerves) following the administration of two doses of both agents, 2.5 mmol/kg and 0.6 mmol/kg.

Results: Rats injected with gadodiamide and gadoterate meglumine showed no significant changes in the spatial working memory performance as compared to baseline and control groups. Moreover, no statistically significant alteration in the number of proliferating $BrdU^+$ cells and newly maturing neurons (co-labeled $BrdU^+/NeuN^+$ cells) in the dentate gyrus of the hippocampus was observed following Gd brain deposition. However, rats exposed to Gadodiamide (Omniscan) showed a noticeable decreasing trend in both, the rate of hippocampal neurogenesis and the behavioral outcome one month after the last GBCA injection. All GBCAs used resulted in significant Gd metal deposition in central and peripheral nervous tissues, with the highest concentrations being detected following Gadodiamide administration.

Conclusions: Our findings indicate that Gadolinium retention in the brain does not affect hippocampal neurogenesis or alter working memory function in young rats. Nevertheless, the effect of Gadodiamide exposure on hippocampal related functions and neurogenesis requires further investigation due to the decreasing trend observed. More importantly, this study provides the first evidence for Gd deposition in the spinal cord and peripheral nerves after exposure to linear and macrocyclic GBCAs. Such metal deposition might underlie the pathophysiology of abnormal sensory symptoms and pain reported by some patients following GBCAs administration. Further research is required to assess the impact of such Gd deposition on sensory and motor neuronal activities.

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ABBREVIATIONS

AQP4	Aquaporin-4 water channel
BBB	Blood brain barrier
BrdU	Bromodeoxyuridine/ 5-Bromo-2'-deoxyuridine
BSA	Bovine Serum albumin
CA3	Cornu Ammonis 3 region
CE-MRI	Contrast-enhanced magnetic resonance imaging
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DG	Dentate Gyrus
DN	Dentate Nucleus
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
GBCA	Gadolinium Based Contrast Agent
GCs	Granule Cells
GCL	Granular Cell Layer
Gd	Gadolinium metal
Gd^{+3}	Gadolinium ion
GDD	Gadolinium deposition disease
GP	Globus Pallidus
HCL	Hydrochloric acid
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry

i.p.	Intraperitoneal
ISF	Interstitial fluid
LD_{50}	Median Lethal Dose
LOQ	limit of quantification
ML	Molecular Layer
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NeuN	Neuclear Neuronal Antigen
NGS	Normal Goat Serum
NPs	Neural Progenitors
NSCs	Neural Stem Cells
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
PNS	Peripheral Nervous system
RGL	Radial Glia-like cells
SGZ	Sub granular zone
SI	Signal intensity
SVZ	Subventricular Zone
TEM	Transmission Electron Microscopy
TEM-EDX	Transmission Electron Microscopy coupled to Energy Dispersive X-ray Spectroscopy
VRS	Virchow-Robin space

CHAPTER I

INTRODUCTION

A. Gadolinium-Based Contrast Agents

Gadolinium-based contrast agents (GBCAs) are chemical substances that are used clinically to enhance the visualization of internal organs on magnetic resonance imaging (MRI). With their first introduction in 1982 at the annual meeting of the Radiological Society of North America, GBCAs have transformed and revolutionized diagnostic medical imaging till the present day (Runge et al., 1983). In 1988, the first GBCA got approved for clinical use by the US Food and Drug Administration (FDA), and currently there are nine agents that are commercially available (Lohrke et al., 2016; Rozenfeld & Podberesky, 2018). It is estimated that approximately 40% of the performed MRI scans require the use of GBCAs, with 30 million intravenous administrations being given annually worldwide (Layne, Dargan, Archer, & Wood, 2018; Rozenfeld & Podberesky, 2018).

1. Clinical Indications

GBCAs are used clinically for several medical conditions ranging from neoplastic, inflammatory, infectious, and vascular disorders across the different anatomical body regions. Contrast-enhanced MRI (CE-MRI) is a significant non-invasive and radiation free imaging modality that is effective not only in the initial diagnosis and detection of diseases, but also in the assessment process, disease staging, biopsy guidance, therapeutic management, and follow up measures (Ramalho,

Ramalho, Semelka, & Castillo, 2016; Soares, Lequin, & Huisman, 2017). Intravenous contrast media administration is nowadays more frequently used in the assessment of the brain, the spinal cord, the abdominal viscera, the breast, the musculoskeletal system, and in cardiac imaging and MR angiography (Lohrke et al., 2016). Moreover, it is considered an essential diagnostic tool for central nervous system (CNS) pathologies due to its ability to investigate areas of blood brain barrier (BBB) disruption in patients with neurological disorders such as primary and secondary CNS tumors, strokes, vascular malformations and aneurysms, or CNS infections. CE-MRI is also considered the golden standard for detecting and monitoring of multiple sclerosis (MS) inflammatory demyelinating lesions in the brain and the spinal cord (Anzalone, Gerevini, Scotti, Vezzulli, & Picozzi, 2009; Csepany, 2018; Elbeshlawi & AbdelBaki, 2018; Essig et al., 2012; Holowka, Shroff, & Chavhan, 2019; Lohrke et al., 2016; Saade et al., 2018).

2. Mode of Action

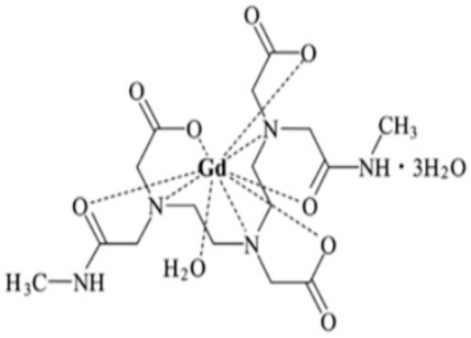
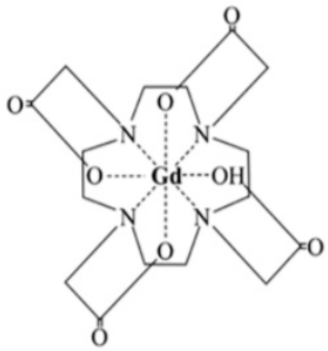
GBCAs are a family of organic compounds “polyamino-carboxylic acids” that are chelated to Gadolinium (Gd), a rare heavy earth metal that belongs to the Lanthanide group. The Gd metal is characterized by seven unpaired electrons (Holowka et al., 2019; Layne et al., 2018), as such, its highly paramagnetic and results in an increased delineation between normal and abnormal structures by shortening the T1 and T2 relaxation times of adjacent water protons in the body tissues (Holowka et al., 2019; Layne et al., 2018; D. H. Lee, 1991; Lohrke et al., 2016; Rozenfeld & Podberesky, 2018). This results in an enhancement effect enabling the detection of

diseases relative to normal anatomy, and significantly improved visualization of tissues and anatomical structures.

3. Classification of GBCAs

Gadolinium ion (Gd^{3+}) is toxic in its free form and is therefore chelated to organic ligand molecules that ensure its safe distribution to the intravascular and interstitial spaces, and its rapid elimination by the renal system with an average elimination half-life of 1.5- 2 hours (Aime & Caravan, 2009; Ramalho, Semelka, et al., 2016). GBCAs are classified according to the chemical structure of the carrier molecule into linear and macrocyclic agents. Linear agents are characterized by an "open chain" elongated linear structure of the chelate that does not enclose the Gd ion completely, whereas in macrocyclic agents, the Gd^{3+} is trapped inside the cavity of a "cage-like" structure of the organic ligand (Hao et al., 2012; Layne et al., 2018; Lohrke et al., 2016; Morcos, 2008; Pasquini et al., 2018; Rozenfeld & Podberesky, 2018). Macrocyclic agents are therefore characterized by a higher thermodynamic and kinetic stability, leading to a lower risk of gadolinium dissociation and release into body tissues (Fraum, Ludwig, Bashir, & Fowler, 2017; Frenzel, Lengsfeld, Schirmer, Hutter, & Weinmann, 2008; Karabulut, 2015; Port et al., 2008). Moreover, GBCAs are further sub-classified into ionic agents, which result in charged particles when dissolved, and nonionic agents that remain otherwise neutral (Layne et al., 2018; Morcos, 2008). Among the clinically used GBCAs, Gadodiamide (linear nonionic) and Gadoterate meglumine (macrocyclic ionic) agents will be examined in this study **(Table 1)**.

Table 1. Properties of Gadodiamide and Gadoterate meglumine paramagnetic contrast agents.

	Gadodiamide	Gadoterate Meglumine
Trade Name	Omniscan ®	Dotarem ®
Structure		
Molecular weight	591.7 g/mol	558.6 g/mol
Type	Linear nonionic	Macrocyclic ionic
FDA approval	1993	2013
Manufacturer	GE Healthcare	Guerbet
Clinical Dose	0.1 mmol/kg	0.1 mmol/kg
Half-life (hr)	1.3 ± 0.3 hr	1.4 ± 0.2 hr in Males 2.0 ± 0.7 hr in Females
Biodistribution	Extracellular	Extracellular
Protein binding	No	No
Excretion	Renal	Renal
FDA approved to clinically assess:	Disrupted vascularity and abnormal lesions in the CNS, the thoracic, abdominal, pelvic and peritoneal cavities.	Regions of BBB disruption and abnormal vascular system in the brain, the spinal cord, and associated tissues.

Data obtained from: *(Holowka et al., 2019; Kartamihardja, Nakajima, Kameo, Koyama, & Tsushima, 2016; Layne et al., 2018; Lyapustina, Goldfine, Rhyee, Babu, & Griswold, 2019; Rozenfeld & Podberesky, 2018)*

4. Gadolinium Deposition in the Brain

GBCAs were considered safe and well tolerated agents with a low rate of adverse allergic and anaphylactic reactions (0.079- 0.096 %) (Jung et al., 2012; Rogosnitzky & Branch, 2016), until recent scientific evidence showed that Gd metal is depositing in the skin, bones, liver, and the brain in patients with normal renal function (Lohrke et al., 2017; Maximova et al., 2016; R. J. McDonald, J. S. McDonald, D. Dai, et al., 2017; Murata, Gonzalez-Cuyar, et al., 2016; Murata, Murata, Gonzalez-Cuyar, & Maravilla, 2016; Roberts, Lindhorst, et al., 2016). In 2014, Kanda et al. first reported the clinical observation of an increased signal intensity (SI) in the dentate nucleus (DN) and globus pallidus (GP) on unenhanced MRI in patients with previous exposure to GBCAs, indicating Gd retention (Kanda, Ishii, Kawaguchi, Kitajima, & Takenaka, 2014). This was followed by multiple preclinical and clinical MRI imaging studies that confirmed the observation in patients receiving linear GBCAs, but not macrocyclic agents that are characterized by a higher in-vivo stability (Adin et al., 2015; Cao, Huang, Shih, & Prince, 2016; Errante et al., 2014; Jost et al., 2016; Kanda, Osawa, et al., 2015; J. Y. Lee et al., 2017; McDonald et al., 2015; A. Radbruch et al., 2017; A. Radbruch et al., 2015; Ramalho et al., 2015; Robert et al., 2015; Robert et al., 2016; Weberling et al., 2015). Subsequently, post-mortem evaluation of human brain autopsies from patients that received contrast agents confirmed that all types of GBCAs, both linear and macrocyclic, result in Gd retention in the brain interstitium in varying amounts (Kanda, Fukusato, et al., 2015; J. S. McDonald et al., 2017; McDonald et al., 2015; R. J. McDonald, J. S. McDonald, D. F. Kallmes, et al., 2017; Murata, Gonzalez-Cuyar, et al., 2016; Zhang, Cao, Shih, Hecht, & Prince, 2017). Gadolinium deposits were found to be mainly concentrated in the globus pallidus and the dentate nucleus of

the cerebellum, but it was also detected at a lower degree in other brain areas such as the cerebellar white matter, cerebral white matter, frontal lobe cortex, the substantia nigra, the thalamus and pons (Zhang et al., 2017). Quantification of Gd deposits using inductively coupled plasma mass-spectrometry (ICP-MS) in human neuronal tissue revealed a metal concentration ranging between 0.1-58.8 μg/g, with a significant dose dependent pattern of deposition (McDonald et al., 2015). Furthermore, macrocyclic agents were found to deposit approximately 20 times lower than linear GBCAs (Murata, Gonzalez-Cuyar, et al., 2016). Transmission electron microscopy (TEM) showed that Gd deposits are mainly localized in the endothelial walls of cerebral capillaries, in the neuronal interstitium, and within neuronal cells nuclei. Moreover, microscopic examination of brain specimens revealed no gross histopathological changes or abnormal neuronal morphology or cellular injury in regions of Gd deposits (Kanda, Fukusato, et al., 2015; Lohrke et al., 2017; J. S. McDonald et al., 2017; McDonald et al., 2015; R. J. McDonald, J. S. McDonald, D. F. Kallmes, et al., 2017; Murata, Gonzalez-Cuyar, et al., 2016; Zhang et al., 2017). Intracranial Gd retention phenomenon was further confirmed in patients without intracranial abnormalities or disrupted BBB (R. J. McDonald, J. S. McDonald, D. F. Kallmes, et al., 2017). It has also been recently confirmed in the pediatric population (Flood, Stence, Maloney, & Mirsky, 2017; Kasper, Schemuth, Horry, & Kinner, 2018; J. S. McDonald et al., 2017; Miller, Hu, Pokorney, Cornejo, & Towbin, 2015; Roberts, Chatterjee, et al., 2016; Roberts & Holden, 2016; Roberts, Welsh, LeBel, & Davis, 2017; Rossi Espagnet et al., 2017; Ryu et al., 2018; Schneider et al., 2017; Tibussek et al., 2017; Young et al., 2018). Change in the brain MRI SI in children was detected following as low as two GBCA injections (Hu, Pokorney, Towbin, & Miller, 2016).

5. Mechanism of Gadolinium Deposition in the Brain

- ***Entering the Brain***

The biodistribution of GBCAs and the mechanism of Gadolinium retention in the brain parenchyma became a central research topic especially after the post-mortem human brain confirmatory studies. Researchers tried to explain the path that led these agents that were previously considered to have only an extracellular distribution in the body (Aime & Caravan, 2009) and not known to cross the BBB (Guo, Yang, & Zhang, 2018; Morcos, 2008) to end up causing brain deposition even in people without intracranial abnormalities (Kanda, Fukusato, et al., 2015). In fact, one of the major indications of GBCAs use in the clinic is the investigation of BBB disruption in patients with neurological diseases (Kanal & Tweedle, 2015). This led to several hypotheses that have been proposed in an attempt to explain the mechanism underlying this phenomenon.

a. <u>The Transmetallation Hypothesis</u>

In 2016, Kanda et al. hypothesized that GBCAs are capable of partially crossing the intact BBB through metal transporters (Kanda, Nakai, et al., 2016; Kanda, Oba, Toyoda, Kitajima, & Furui, 2016). That assumption was made after examining postmortem brain samples of patients that received linear agents, and detecting clusters of Gd deposits in the endothelial wall of cerebral vessels using TEM coupled to energy dispersive X-ray spectroscopy (TEM-EDX) (Kanda, Fukusato, et al., 2015). Researchers postulated that the distribution of Gd deposits across the BBB might be the result of the in-vivo de-chelation process of non-stable GBCA complexes, followed by

a transmetallation process in the presence of a metal transporter such zinc transporter or other divalent transporters that would facilitate the passage to the brain (Kanda, Nakai, et al., 2016; Pasquini et al., 2018; Taoka & Naganawa, 2018). It was indicated that metal transporters in the endothelial wall are not necessarily specific for a certain metal, but might be also capable of transporting metals with similar chemical characteristics (Bressler et al., 2007; Prybylski, Maxwell, Coste Sanchez, & Jay, 2016). According to this hypothesis, endogenous cations such as zinc, copper, iron or calcium might possibly compete with the Gd ion to bind the chelate structure. Consequently, free Gd^{3+} ions might end up alternatively binding endogenous anions such as carbonate and phosphate, resulting in the formation of insoluble precipitates in the brain tissue (Layne et al., 2018; Morcos, 2008).

Nevertheless, this hypothesis is not well supported with scientific evidence and further pre-clinical and clinical research is required. In fact, some studies indicated that endogenous metals are actually transported to the brain via the cerebrospinal fluid (CSF) rather than by crossing the BBB (Aoki, Wu, Silva, Lynch, & Koretsky, 2004; Takeda, Akiyama, Sawashita, & Okada, 1994). Moreover, the transmetallation hypothesis does not explain the passage and deposition of macrocyclic GBCAs that remain intact and stable in-vivo and do not undergo the de-chelation process (Frenzel et al., 2008; Morcos, 2008). Hence, this renders the hypothesis inadequate and insufficient to holistically explain the phenomenon of Gd deposition in the brain.

b. <u>The Glymphatic System Pathway</u>

On the other hand, scientists indicated recently that GBCAs might alternatively cross the Blood-CSF barrier, and the Glymphatic system was proposed as a potential

path to reach the brain (Pasquini et al., 2018; Taoka, Jost, Frenzel, Naganawa, & Pietsch, 2018; Taoka & Naganawa, 2018). The Glymphatic system was first described in 2012 by Iliff et al. as a system responsible for the clearance of interstitial metabolic wastes from the brain (Iliff et al., 2012). The glymphatic system nomenclature is derived from the term "*glial*", referring to the astroglial cells that surround the perivascular spaces and "*lymphatic*", referring to the lymphatic-like function of this system in the drainage of extracellular matrix (ECM) waste materials such as lipids and proteins out of the brain (Rozenfeld & Podberesky, 2018; Taoka et al., 2018; Taoka & Naganawa, 2018).

According to this pathway, intravenously injected GBCAs distribute through the systemic blood circulation and then cross the blood-CSF barrier at the level of the choroid plexus (Jost et al., 2017; Pasquini et al., 2018). Unlike the brain vasculature, the choroid plexus epithelial cells are devoid of tight junctions and are characterized by being fenestrated, representing a site of a weak barrier and allowing low-molecular weight molecules such as GBCAs to cross (Iliff et al., 2013; Jost et al., 2017; Pullicino, Radon, Biswas, Bhojak, & Das, 2018; Saade et al., 2018). GBCAs are then carried in the CSF flow through the ventricular system by passing from the lateral ventricles to the third ventricle through the interventricular foramen of Monro. The flow then continues to the fourth ventricle via the cerebral aqueduct, and ultimately enter the subarachnoid space of the CNS via the central foramen of Magendie and the two lateral foramina of Lushka (**Figure 1**) (Plog & Nedergaard, 2018). The bulk flow of CSF containing GBCAs move in the subarachnoid space and enter to the brain through the perivascular spaces that surround the penetrating cerebral arteries of the brain parenchyma. The perivascular spaces, which are also referred to as the Virchow-Robin spaces (VRS), are

surrounded by astrocytic end-feet that create a conduit like well-structured passages (Jessen, Munk, Lundgaard, & Nedergaard, 2015; Pasquini et al., 2018; Plog & Nedergaard, 2018). Accordingly, the VRS space is anatomically enclosed between the basement membrane of the penetrating blood vessels of the brain and the astrocytic end-feet structures that constitute its external wall (**Figure 2**). Once in the perivascular space, the subarachnoid CSF containing GBCAs can flow into the brain interstitium by bulk flow movement through the aquaporin 4 water channels (AQP4), which are highly expressed on astrocytic end feet. The perivascular AQP4 channels constitute approximately ≈ 50% of the surface area of astroglial end feet and hence create a low resistance path that facilitates the influx of CSF into the interstitium (Iliff et al., 2012). Moreover, the constituents of the CSF can also potentially pass through the intercellular cleft between the end feet processes of astrocytes that measures approximately ≈ 20nm (Iliff et al., 2012). As reported by Nedegraf et al., solutes with a molecular weight of less than 100 kD can actually pass between the end-feet of astrocytes, indicating that all types of GBCAs can also consequently cross (Jessen et al., 2015; Nedergaard & Goldman, 2016; Pasquini et al., 2018).

GBCAs in the subarachnoid CSF enter the brain interstitium, interact with the extracellular environment and potentially deposit. Subsequently, they are potentially cleared with the interstitial fluid (ISF) along the peri-venous spaces. The flow of CSF and its constituents is eventually drained back to the venous blood through the arachnoid villi structures that are located in the dural venous sinuses (Plog & Nedergaard, 2018). Additionally, CSF drainage out of the brain can also occur through a system of meningeal lymphatic vessels that align the venous sinuses, continuing to the

cervical lymph nodes and eventually excreted to the venous blood (Aspelund et al., 2015; Louveau et al., 2015; Plog & Nedergaard, 2018).

The driving force of the CSF flow that carries GBCA molecules along the glymphatic pathway and the directionality from the peri-arterial space to the interstitium, where it interacts with the ECM and back to the peri-venous space is attributed to several physiological factors. These factors include: the arterial pulsation and systemic blood pressure, the CSF pressure gradient, and the increased AQP4 water channels expression on the astrocytic end-feet surrounding the peri-venous spaces, as compared to the peri-arterial spaces (Iliff et al., 2012; Saade et al., 2018). All these factors contribute in creating a low resistance path for CSF and accompanying GBCAs to flow in an arterio-venous direction.

Research indicated that the glymphatic system is not only involved in the entrance mechanism of GBCAs, but also in the slow long-term excretion of Gd deposits from the brain (Jost et al., 2018; Kanda et al., 2017; Kartamihardja et al., 2016). Even though the glymphatic pathway provides an insight of GBCAs' biodistribution in the CNS, it does not explain the non-uniform distribution of Gd precipitates in the brain. Several questions remain to be answered such as when exactly the Gd deposition occurs or why the deposits are concentrated in certain brain regions. The exact dynamics of how GBCAs interact with the brain tissue is still not clearly understood and remains to be determined.

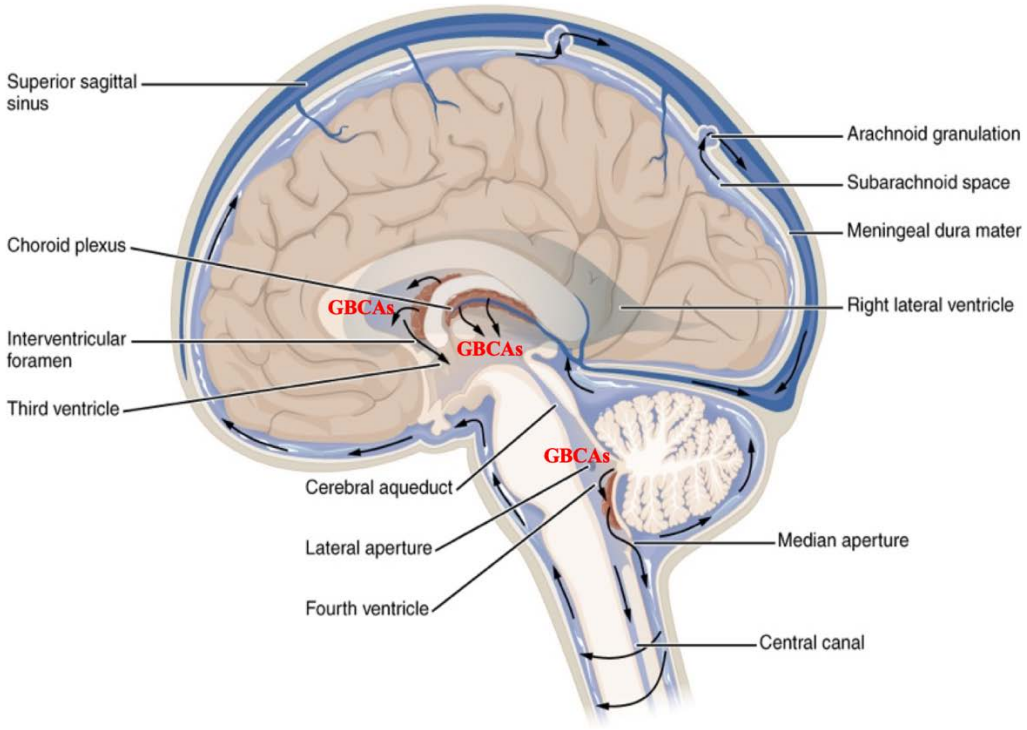


Figure 1. GBCAs entry to the brain through the CSF. GBCAs are low-molecular weight molecules that cross the blood-CSF barrier at the level of the choroid plexus. GBCAs are then carried in the CSF flow through the ventricular system and ultimately enter to the subarachnoid space of the brain.

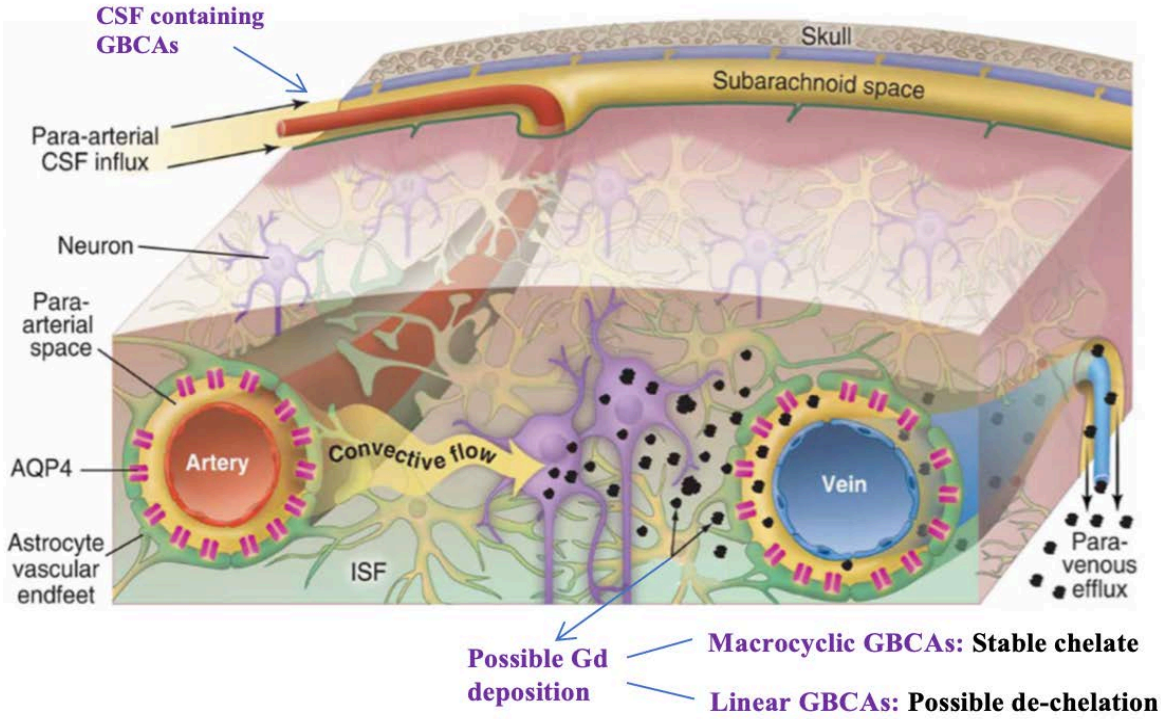


Figure 2. Schematic Representation of the Glymphatic Pathway. The CSF-ISF exchange as a potential entry path of GBCAs to the brain parenchyma. *Adapted from: (Plog & Nedergaard, 2018)*

6. Types of Gadolinium Deposits in the Brain

- ***Chelated vs de-chelated form***

Scientists attempted to determine the nature of residual Gd deposits considering the diverse physiological environment that GBCAs might interact with. Although the exact chemical composition and identity of Gd-containing deposits are still unknown and not fully understood, several preclinical studies were able to reveal some of its characteristics. A bioanalytical study conducted recently in 2017 by Frenzel et al. evaluated the chemical form of Gd deposits in rat brains three days and 24 days after the administration of several types of linear and macrocyclic GBCAs, including

Gadodiamide and Gadoterate meglumine (Frenzel et al., 2017). In order to identify the type and speciation of the deposits, tissue fractionation was used and Gd metal concentration was measured in soluble and insoluble tissue fractions of brain homogenates using ICP-MS. The study concluded that brain deposits resulting from macrocyclic GBCAs' exposure were almost entirely in a water-soluble form, with a low molecular weight of less than 900 daltons (Da) **(Figure 3).** Researchers indicated that such molecular weight reflects the intact chelated GBCA form, revealing that the Gd metal did not dissociate from its chelate and macrocyclic GBCAs remained stable in vivo as its initial injected structure. Conversely, the deposits resulting from linear GBCAs administration were of three types: insoluble precipitates, water-soluble small molecules, and soluble macromolecules weighing more than 250-300 kDa (**Figure 3).** For all linear agents tested, the detected insoluble Gd precipitates represented the largest quantified fraction corresponding to approximately ≈ 60% of the Gd in the tissue homogenate. Such findings reflect the de-chelation and dissociation of the Gd metal from the unstable linear ligand structure in vivo. In fact, such insoluble precipitates are the particles responsible for the increase in SI on un-enhanced T1-weighted MRI imaging in patients exposed to linear GBCAs, reflecting the de-chelation process (Frenzel et al., 2017).

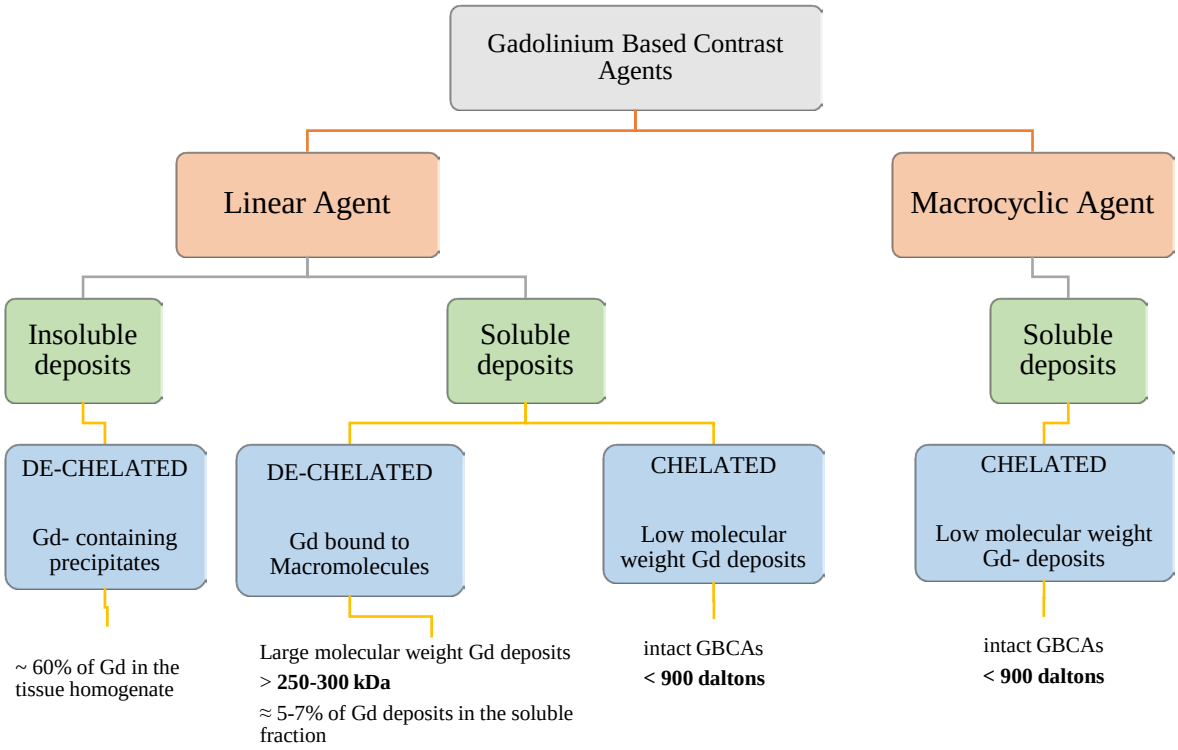


Figure 3. **Types of Gadolinium Deposits in the Brain.** Schematic representation of the different chemical species of Gadolinium deposits in the rat brain tissue after receiving multiple linear and macrocyclic GBCAs intravenous injections. *Data obtained from: (Frenzel et al., 2017; Gianolio et al., 2017)

7. Clinical Significance of Intracranial Gadolinium Retention

Following the confirmation of Gd metal deposition in the brain, several recommendations and policy statements were issued. In 2017, the FDA published a statement indicating that there is no compelling evidence that the use of GBCAs, including linear agents that are associated with high Gd brain deposition result in harmful clinical effects, and hence their use will not be restricted clinically. The FDA recommended healthcare professionals to reduce and limit patients' exposure to GBCAs as possible, without avoiding any essentially required CE-MRI scans, and calling for more research (Food and Drug Administration, 2017). Furthermore, the European

Medicines Agency (EMA) retracted linear GBCAs from the market and suspended their clinical use across Europe, in an attempt to avoid and prevent any potential future risks of Gd tissue retention (European Medicines Agency, 2017). Additionally, the UK Medicines and Healthcare products Regulatory Agency (MHRA) published a statement in December 2017 suspending the licenses for linear GBCAs, except for gadoxetate and gadobenate dimeglumine that have significant diagnostic potential in liver imaging (MHRA, 2017).

Few clinical studies were conducted to assess the potential clinical risks and consequences of intracranial Gd retention. In a large retrospective population-based study, Welk et al. investigated if Gd deposits in the brain, which are mainly located in the globus pallidus and the cerebellar dentate nucleus predispose patients to future motor dysfunction or Parkinson's neurodegenerative disease (Welk et al., 2016). The study reported no significant association between GBCA exposure and the development of Parkinson's disease or Parkinsonian like motor symptoms. However, one of the limitations of the study was the low number of subjects that received four or more GBCA doses. Another clinical study by Mcdonald et al. reported no association between GBCA exposure and neurocognitive decline and impairment (RSNA, 2017). The longitudinal population-based study was conducted at the Mayo Clinic, evaluating 4261 elderly patients that received a mean of two GBCA administrations (range 1- 28 doses). The study concluded that Gd exposure is not a predictor or a significant risk factor for cognitive decline as measured by neuropsychological testing, the clinical dementia rating scale, the Blessed dementia scale, and the mental status exam. Moreover, Gd brain retention was not a critical factor in facilitating the progression from a normal cognitive stage to mild cognitive impairment or dementia. The

researchers also evaluated the motor performance, reporting no significant impairment as measured by the Unified Parkinson's Disease Rating scale.

On the other hand, there are few reports in the literature that linked and attributed adverse clinical symptoms to Gd tissue deposition. The "Gadolinium deposition disease (GDD)" term was first suggested by Smelka et al. in 2016, describing a group of symptoms that appear within hours to several months post GBCA injection in patients with normal renal function (Semelka, Ramalho, Vakharia, et al., 2016). In a clinical study, the researchers conducted physical examination on four patients with normal renal function who developed clinical symptoms following a range of 1-4 GBCA administrations (Semelka, Commander, Jay, Burke, & Ramalho, 2016). All patients reported central trunk pain and peripheral extremity pain, in addition to dermal thickening and clouded mentation. Patients reported their symptoms at short term (2-3 months) and long-term (7-8 years) periods post GBCA exposure, with Gd metal being detected in their urine samples. Moreover, a clinical survey study was conducted in which 42 respondents reported side effects post CE-MRIs ranging from central pain (n=15), peripheral extremity pain (n=26), headache (n=28), bone and joint pain (n=26), skin thickening (n=22), clouded mentation and difficulty in concentration (n=29) (Semelka, Ramalho, Vakharia, et al., 2016; Semelka, Ramalho, AlObaidy, & Ramalho, 2016). The same clinical signs were further reported by another survey that examined the chronic effects of retained Gd, with 17 subjects indicating that their symptoms persisted for more than three months. The patients reported chronic pain including paresthesia, electric-like feeling, burning sensation, and deep bone pain (Williams & Grimm, 2014). Other survey studies reported headache and bone and joint pain as the most common symptoms following CE-MRIs (Burke et al., 2016). However,

the clinical significance of such survey studies is questioned as they were not based on objective clinical examination or medical records, but rather depended mainly on self-reported symptoms and the subjective identification of the condition. Moreover, the GDD condition is still to date not established or validated by other researchers (Lyapustina et al., 2019).

The controversy and uncertainty remains regarding GBCAs safety, with no yet definite conclusions and proven causality between Gd tissue retention and toxicity. Further research and well-designed studies are required to assess the potential risk and unknown long-term effects of residual Gd, especially in the brain. Attention must be driven towards promoting the safety of high-risk groups such as patients with medical conditions requiring multiple CE-MRIs and children, considering the vulnerability of the developing brain.

B. The Neurogenic Potential of the Brain

Adult neurogenesis is the process of the continuous generation of new neurons from neural stem cells (NSCs) and their functional integration into pre-existing circuitries in the adult mammalian brain (Baptista & Andrade, 2018; Toda & Gage, 2018). This phenomenon was for long questioned and faced with skepticism among the scientific community, in which neural generation was thought to occur only prenatally during brain development. In the 1960s, the first evidence of postnatal neural regeneration and cellular proliferation in the mammalian brain was provided by Altman et al. (Altman, 1963; Altman & Das, 1965). Moreover, the existence of adult neurogenesis in the human brain was first reported by Eriksson et al. through detecting proliferative neural progenitor cells in postmortem brain samples of cancer patients

(Eriksson et al., 1998). Subsequent confirmation of this finding in humans was further provided by several studies that used C_{14} dating and immunohistochemical analysis (Boldrini et al., 2018; Knoth et al., 2010; Moreno-Jimenez et al., 2019; Spalding et al., 2013).

Adult neurogenesis process occurs in two main regions of the brain, which are known as the classical neurogenic niches: the sub-ventricular zone (SVZ) of the lateral ventricles and the dentate gyrus of the hippocampus (Oyarce, Bongarzone, & Nualart, 2014). Moreover, recent evidence have shown that neurogenesis also occurs in other regions of the brain, known as the "unconventional niches" including the hypothalamus, the substantia nigra, the amygdala, the cerebellum, and the spinal cord (Cheng, 2013; Fowler, Liu, & Wang, 2008; A. Lee et al., 2005; Lie et al., 2002; Obermair, Schroter, & Thallmair, 2008; Oyarce et al., 2014). However, the neurogenic potential in these unconventional niches was reported to occur at a lower rate and with a limited neuronal migration capacity (Oyarce et al., 2014).

C. Adult Hippocampal Neurogenesis

Among the different brain regions with neurogenic capacity, the hippocampus represents the most studied and significant unique structure. Adult hippocampal neurogenesis is a dynamic process that occurs in a specific anatomical region of the dentate gyrus known as the sub-granular zone (SGZ). The dentate gyrus (DG), which is attributed as the main input region of the hippocampus, is morphologically divided into three distinct layers: the outer molecular layer, the granular cell layer, and the inner sub-granular zone (Toda & Gage, 2018). The granular cell layer (GCL) of the DG houses

the cell bodies of the mature granule neuronal cells (GCs), which are characterized by being highly condensed and express the mature neuronal differentiation marker (NeuN). Moreover, the molecular layer (ML) contains the dendrites of the mature granule cells, in addition to terminal axons that originate and project from different regions of the brain. Finally, the inner SGZ represents the germinal layer of the hippocampus which contains the adult NSCs niche, and is anatomically located between the GCL and the hilar zone.

During the neurogenesis process, NSCs pass through different stages including proliferation, differentiation, maturation, and finally synaptic integration into the existing neural network of the DG. Adult NSCs in that region, which are also known as the Radial Glia-like (RGL) cells or Type 1 cells, self-renew and give rise to intermediate neural progenitors (NPs), which further differentiate into neuroblasts and finally mature into granule cells **(Figure 4)** (Toda & Gage, 2018). The newly born neurons that survive will functionally integrate into the hippocampal network by sending projections to the Cornu Ammonis (CA3) region, and by receiving excitatory synaptic input from the mature GCs, the lateral entorhinal cortex, and the CA3 pyramidal neurons (Vivar et al., 2012; Vivar & van Praag, 2013).

Numerous studies and accumulating evidence have shown that adult neurogenesis in the dentate gyrus contributes to hippocampal-dependent cognitive functions such as learning and memory, spatial navigation, and mood regulation (Baptista & Andrade, 2018; Dupret et al., 2008; Lazarov & Hollands, 2016; Saxe et al., 2006; Snyder, Hong, McDonald, & Wojtowicz, 2005; Toda & Gage, 2018; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006; Yau, Li, & So, 2015). However, the exact nature of that neurobiological contribution is still under scientific investigation. Newly

DG born cells have been shown to play a crucial role in pattern separation and preventing interference between similar inputs in newly formed memories (Baptista & Andrade, 2018; Sahay et al., 2011; Yau et al., 2015). Moreover, the disruption and dysregulation of this process has been implicated in the pathology of several neurodegenerative and psychiatric disorders including the Alzheimer's disease, dementia, Parkinson's disease, temporal lobe epilepsy, depression, anxiety and mood disorders (Apple, Fonseca, & Kokovay, 2017; Kang, Wen, Song, Christian, & Ming, 2016; Toda, Parylak, Linker, & Gage, 2019).

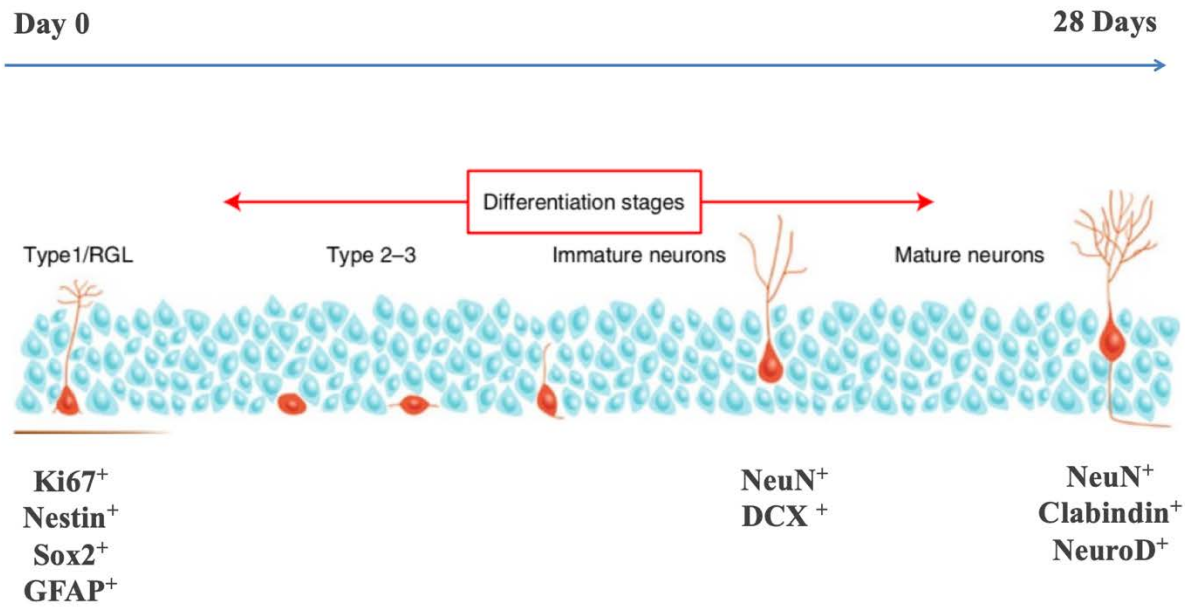


Figure 4: Differentiation Stages of Neural Stem Cells in the Dentate Gyrus of the Hippocampus. *Adapted from: (Moreno-Jimenez et al., 2019)*

D. Aim of the Study

While the clinical significance of gadolinium deposition in the brain remains unsettled, it does however, raise important questions concerning its long-term consequences on learning and memory in developing brains of children undergoing multiple CE-MRIs. Therefore, the purpose of the present study is three-fold. The first is to assess whether Gd brain deposition affects cognitive functions such as the working memory performance. The second aim is to investigate whether multiple exposures to linear and macrocyclic GBCAs at young age have an impact on the stem cell niche in the hippocampal formation. And lastly, to investigate if exposure to GBCAs leads to Gd deposits in the spinal cord and peripheral nerves.

CHAPTER II

MATERIALS AND METHODS

A. Animals

Male Sprague Dawley rats, weighting 140-150 g at the start of the experiments, were used in the experiments of this study and all the experimental procedures were conducted in accordance with the ethical guidelines and under the approval of the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut. All laboratory animals were housed in standard environmental conditions with a controlled temperature range (20-22 ^{o}C) and a 12 hours light/dark cycle. Animals were provided with water and standard rodent chow *ad libitum*, with health assessment and body weight measurement performed daily.

B. Experimental Design

Two independent experiments were performed, one to assess the effect of Gd brain deposition on hippocampal neurogenesis and working memory function, and another tissue quantification experiment to assess the Gd metal concentration in the central and peripheral nervous system (PNS) following exposure to GBCAs.

1. GBCA exposure

Two types of GBCAs were tested in this preclinical study, Gadodiamide and Gadoterate Meglumine; which are the most commonly used MRI enhancement agents

in Lebanon. Young male Sprague Dawley rats (Age: 4 weeks old, weight: 140-150 g) were randomly divided into four experimental groups:

Group 1: received intraperitoneal (*i.p*) injections of Gadodiamide (Omniscan; linear non-ionic GBCA; GE Healthcare) at a dose of 2.5mmol/kg/injection.

Group 2: received *i.p* injections Gadoterate Meglumine (Dotarem; macrocyclic ionic GBCA; Guerbet) at a dose of 2.5 mmol/kg/injection.

Group 3: received *i.p* injections of 0.9% Normal Saline and acted as a control.

Group 4: Naïve rats that received no treatment, only BrdU injections in order to serve as a reference for the normal rate of proliferating stem/progenitor cells and hippocampal neurogenesis in the brain.

The contrast agents and saline were injected intraperitoneal for 20 consecutive days **(Figure 5).** The dose of the contrast agents (2.5 mmol/kg) is equivalent to four times the clinically used dose after the adjustment for species differences and body surface area as recommended by the FDA (FDA Center for Drug Evaluation and Research, 2005). Animals (n=5 per group) were sacrificed at two different time points (at day 22 and day 48 of the experiment) for the assessment of cellular proliferation and neuronal maturation in the DG of the hippocampus respectively (**Figure 5**).

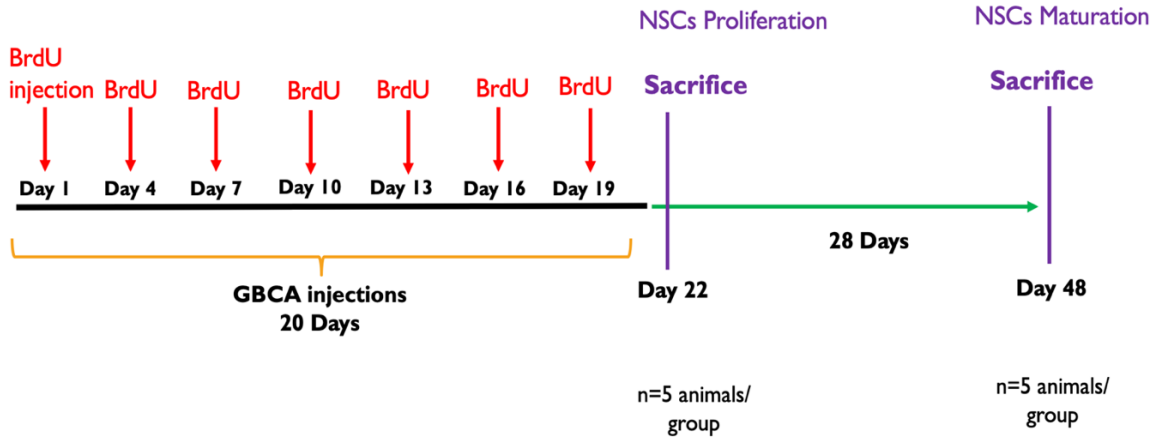


Figure 5. Experimental Design. The experimental timeline of GBCA injections (20 consecutive days), BrdU injections (every 3 days), and sacrifice time points (at day 22 and day 48). *NSCs: Neural stem cells*

2. BrdU Administration

In order to assess cellular proliferation and neurogenesis in the hippocampus of the brain, 5-Bromo-2'-deoxyuridine (BrdU) was administered to all the experimental groups. BrdU is a synthetic Thymidine analogue that gets incorporated into the deoxyribonucleic acid (DNA) of dividing cells during the S-phase of the cell cycle. It acts by binding to the Adenine nucleotide of the dividing chromosomes during cell division (mitosis), and thus labeling all proliferating cells in the body. BrdU powder (48.6 mg/kg/injection, Sigma-Aldrich) was weighed and mixed with warm 0.9% normal saline (300 µl/injection). The solution was mildly heated until the BrdU powder becomes completely dissolved. All experimental rats received BrdU injections intraperitoneally every three days throughout the GBCA exposure duration at a dose of 48.6 mg/kg/injection, which corresponds to a total of seven injections with a total dose of 300mg/kg per animal (**Figure 5).** Such dose is an optimal non-toxic dose that ensures maximal labeling of proliferating cells in the brain (Wojtowicz, M., & Kee, 2006).

3. Behavioral Testing: The Spontaneous Alternation T-maze Test

In order to assess the spatial working memory performance and the hippocampal function of the animals, the Spontaneous Alternation T-maze test was performed on all the experimental groups before the treatment to obtain baseline values, at day 10 and day 20 during the GBCA exposure period, and one month after the last GBCA injection (**Figure 6**).

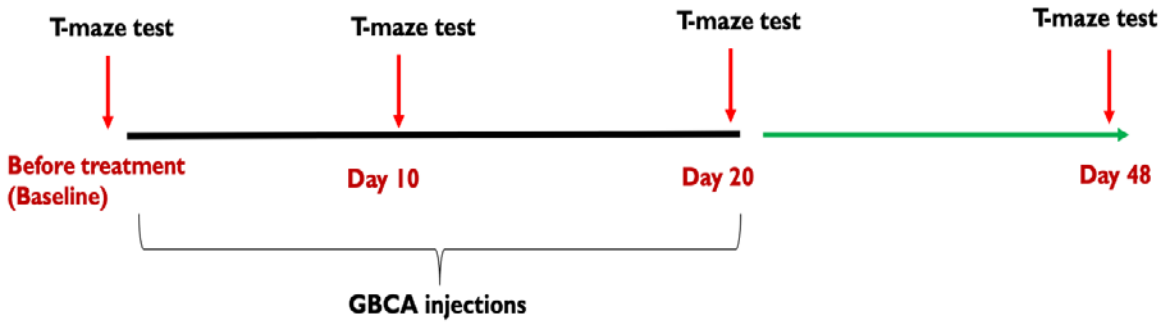


Figure 6. Experimental Timeline of the spontaneous alternation T-maze test performance throughout the experiment.

The spontaneous alternation T-maze test performed in this study is in accordance with the Nature protocol described by Deacon and Rawlins (Deacon & Rawlins, 2006). Animals were transferred to the experimental room and kept initially for 15 minutes in order to accommodate to the new testing environment. No habituation is required for this test, since the novelty of the maze is what drives the animals to spontaneously explore. The test apparatus is T-shaped and composed of three arms, one starting arm and two goal arms with a central partition that is placed extending to the

start arm as shown in **Figure 7A**. Each trial of the T-maze test is composed of two phases: a sample phase and a choice phase. In the first, so called sample phase: the rat is placed in the starting arm at the base of the T-maze and is allowed to choose freely between the right and the left goal arms. The criterion for arm choice is the complete entrance of the animal including the tip of its tail. Once the rat chooses a goal arm by entering it completely, a plastic barrier (door) is used to entrap the animal for a delay time of 30 seconds. In the second phase after the delay time passes, the barrier is removed, and the rat is placed back to the original starting position at the base of the T-maze and is allowed to choose again freely between the two open right and left arms.

With an intact hippocampal function and spatial working memory, the rat would instinctively alternate and choose the opposite arm in an attempt to explore the new environment for potential resources such as food, water, mate or a shelter. On the contrary, the rat is considered to fail a trial if it enters back again to the same arm, indicating that the animal did not remember that it already explored the previously visited arm (**Figure 7B**). Three trials per animal were performed, with each trial requiring approximately 1-2 minutes to be completed. The T-maze apparatus was thoroughly cleaned with 10% alcohol solution between trials in order to mask odors that can act as a confounding variable affecting the animals' behavior and arm choice. The percentages of the successful trials performed per animal were calculated and the resulting data were compared among the different experimental groups at different time points.

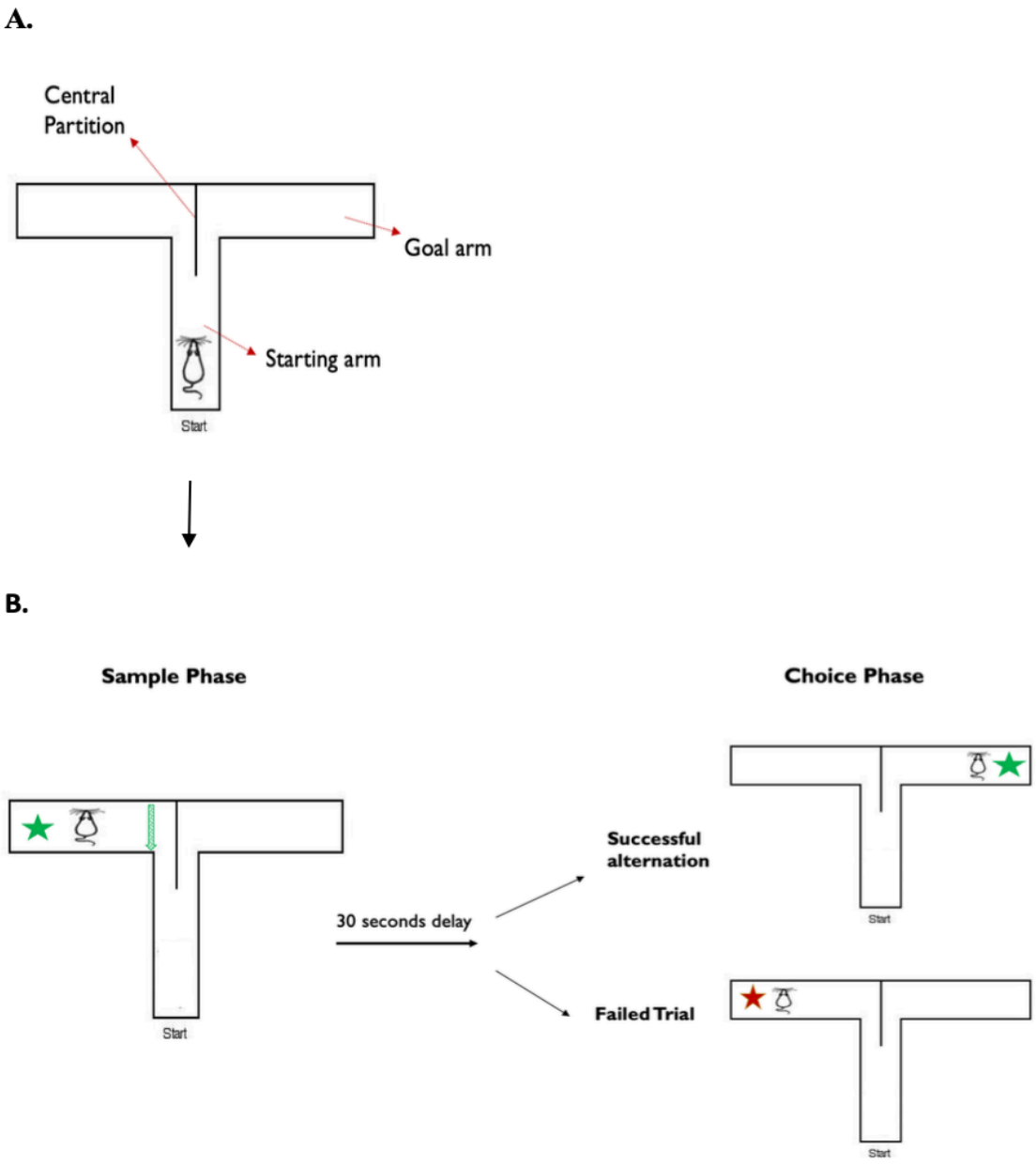


Figure 7. The T-maze Behavioral Test. (A) The T-maze test apparatus. (B) The sample and choice phases of spontaneous alternation T-maze test for the assessment of spatial working memory and cognitive function in rodents.

4. Animal Euthanasia

Animals were sacrificed at two different time points. At 48 hours post the last BrdU injection (day 22) for the assessment of stem/progenitor cells in the hippocampus, and at 29 days after the last BrdU injection (day 48) for the assessment of hippocampal neurogenesis **(Figure 5).** Animals were deeply anesthetized by the intraperitoneal injection of Ketamine (Ketalar®; 80 mg/kg) and Xyla (Xylazine®; 10 mg/kg), and then euthanized by cardiac perfusion with 200ml of 0.9% normal saline for blood displacement followed by 4% Formalin solution for tissue fixation. The brains were extracted and post fixed in 4% Paraformaldehyde (PFA) solution overnight, then transferred to 30% sucrose in 0.1M Phosphate buffer saline (PBS) solution for dehydration and cryoprotection. The brains were stored at 4 °C for approximately three days until complete impregnation.

5. Brain Sectioning: Collection of the Dentate Gyrus of the Hippocampus

Coronal sectioning of the perfused brains was performed using a freezing cryostat microtome. The entire dentate gyrus region of the hippocampal formation was collected, which is an area extending from the stereotaxic coordinates 2.12 mm to 6.6 mm posterior to bregma according to the rat brain atlas (Paxinos & Watson, 1998). Coronal tissue sections of 40μm thickness were serially collected following the Fractionator principle, which is an unbiased stereology method that results in systemic random sampling of brain sections (Gundersen, Jensen, Kieu, & Nielsen, 1999; Schmitz et al., 2014). The DG of the hippocampus was topographically divided into three regions: the rostral, the intermediate and the caudal region. Tissue sections were collected according to the following rostro-caudal coordinates, with the rostral

hippocampal region extending from -2.12 to -3.7 mm in reference to bregma, the intermediate region extending from -3.7 to -4.9 mm, and the caudal extending from -4.9 to -6.3 mm **(Figure 8)** (Chamaa et al., 2018; Paxinos & Watson, 1998). Sections of each of the three regions of the rodent's hippocampus were distributed serially over six wells, with the 1st tissue section being placed in the first well, the 2nd section being placed in the second well and so forth till the 7th section was placed back to the first well **(Figure 9).** The process was followed until the entire hippocampal region was collected. Following this method, each well would eventually be representative of an entire topographic region of the hippocampus (Chamaa et al., 2018). All the tissue sections were placed in a 15mM Sodium Azide dissolved in 0.1M PBS solution for long term preservation.

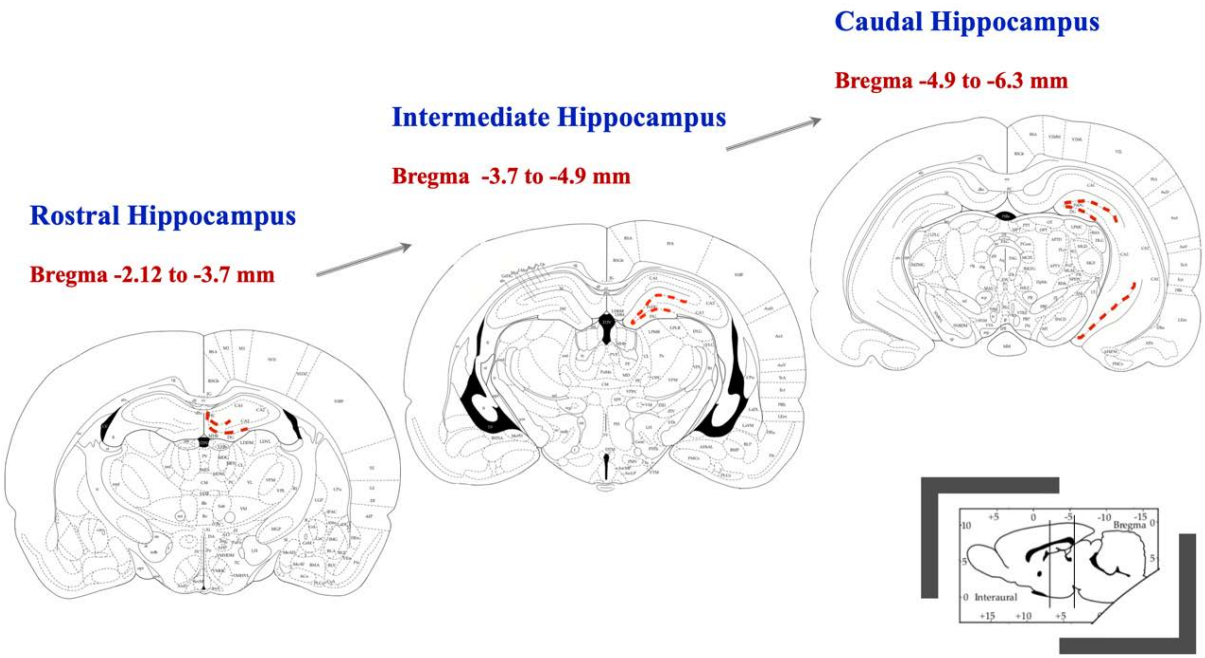


Figure 8. Brain Sectioning and the Stereotaxic Coordinates of the Dentate Gyrus of the Hippocampus. Representative coronal tissue sections of the rostral, intermediate and the caudal topographic regions of the hippocampus (Paxinos & Watson, 1998).

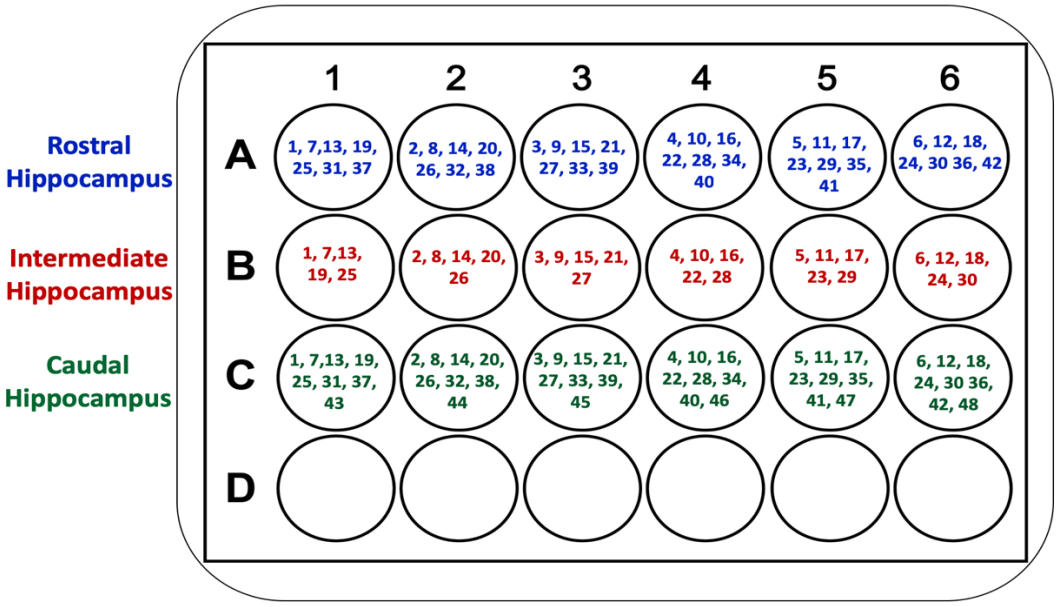


Figure 9. The Fractionator Method. Free floating 40µm coronal brain sections were serially collected in a 24-well plate that includes the three regions of the rodent's hippocampus. The unbiased fractionator method was followed and each well represents every 6th tissue section of a certain topographic hippocampal region. The numbers portrayed in each well indicate the serial distribution of the tissue sections.

6. Immunofluorescent Staining

For each experimental rat, one complete representative well was randomly selected from each topographic region of the hippocampus; the rostral, intermediate, and the caudal regions to be stained for proliferating stem/progenitor cells and mature neurons (**Figure 9**). In order to minimize non-specific binding and cross reaction, the IF staining protocol was performed sequentially over three days. Free floating brain sections were initially washed with 0.1M PBS (Sigma-Aldrich) three times for 5

minutes each. This was followed by a DNA denaturation step, in which the sections are incubated with 2N Hydrochloric acid (HCL) for 30 minutes at 37 °C. Tissue sections were washed again with 0.1M PBS for 5 minutes, and then rinsed with 0.1M Sodium Borate buffer (0.38g/10 ml distilled water; PH 8.5) for 10 minutes at room temperature in order to neutralize the acidic effect. Tissues were then washed 3 times with PBS for 5 minutes each and incubated with 10% Blocking and permeabilizing solution composed of 10% normal goat serum (NGS), 10% bovine serum albumin (BSA), and 0.1% Triton-X diluted in PBS for 1 hour at 4 °C. Hippocampal tissues were then directly incubated with the primary antibody rat monoclonal anti-BrdU (1:100, Bio-Rad) and kept overnight at 4 °C. In the second day, tissues were washed 3 times with PBS for 5 minutes each and then incubated in the dark with the secondary antibody Alexa Fluor-568 goat anti-rat (1:200, Ivitrogen) for 2 hours on a shaker at room temperature. Tissues sections were then washed again 3 times with PBS in order to remove the non-specific binding of the secondary antibody and incubated with the primary antibody that stains mature neurons, mouse monoclonal anti-NeuN (1:400, Millepore, USA) overnight at 4 °C. On the third day of the staining procedure, sections were washed 3 times with PBS for 5 minutes each and stained with the secondary antibody Alexa Fluor-488 goat anti-mouse (1:250, Invitrogen) for 2 hours on a shaker at room temperature. All the primary and secondary antibodies used throughout the staining protocol were diluted in 3% blocking solution composed of 3% BSA, 3% NGS, and 0.1% Triton-X diluted in PBS. Finally, Heochst stain (1:10000, Invetrogen) was added for 10 minutes at room temperature, and tissues were rinsed 3 times with PBS for 5 minutes each. Hippocampal tissues were mounted on glass slides, and coverslips were applied after adding the Anti-Fade mounting medium (Fluoro-Gel, Electron Microscopy Sciences, USA).

7. Cell Quantification and Confocal Microscopy

To quantify the number of stem/progenitor cells in the SGZ of the dentate gyrus of the hippocampus following Gd brain deposition, proliferating $BrdU^+$ cells were counted in the experimental groups of rats that were sacrificed 48 hours after the last BrdU injection. Moreover, to quantify the number of newly maturing neurons in the dentate gyrus of the hippocampus, co-localization with the mature neuronal marker NeuN was assessed and $BrdU^+/NeuN^+$ cells were counted in the experimental groups that were sacrificed 29 days after the last BrdU injection. The $BrdU^+$ cells and the double-labeled $BrdU^+/NeuN^+$ cells were examined and counted using a laser scanning confocal microscope (Zeiss LSM 710) at the 40x-oil objective. The counting was performed exclusively on sections of the chosen representative well from each topographic hippocampal region, and the counted number was multiplied by 6 (the number of representative wells) in order to estimate the full count in each region of the hippocampus. The final number of positive cells in the rostral, the intermediate and the caudal region were then added to obtain the total count in the entire DG of the hippocampus. The total number of proliferating cells and newly maturing neurons in the DG were compared among the different experimental groups at the different sacrifice time points. Confocal Images of BrdU+ cells and $BrdU^+/NeuN^+$ cells were acquired using the Zeiss ZEN 2009 image-analysis software. Tile scan and serial Z-stack images of the dentate gyrus region were taken with maximal intensity projection at 40x-oil objective.

8. Gd Tissue Detection and Quantification using ICP-MS

a. GBCA Exposure and Tissue Collection

For the tissue quantification experiment, another set of young male Sprague Dawley rats (140-150 g) was given serial daily intraperitoneal injections of two types of GBCAs: Gadodiamide (Omniscan) and Gadoterate-Meglumine (Dotarem) for a period of 20 days. Two different doses of both substances were used: the supra-clinical dose (2.5 mmol/kg), and a dose equivalent to the clinically-administered human dose (0.6 mmol/kg) after the adjustment for species differences according to the FDA recommendations (FDA Center for Drug Evaluation and Research, 2005). The control group received saline injections. Rats were scarified one day after the last GBCA injection, and the brains, whole spinal cords, and peripheral nerves (sciatic and trigeminal nerves) were extracted. For trace metal tissue analysis, the epineurium connective tissue layer was removed from the sciatic and trigeminal nerves. Two regions of the extracted brains were collected for metal analysis as shown in **Figure 10**. The first region is the cerebral area extending from the optic chiasm to the midbrain, which is the region covering the hippocampal formation, whereas the second region includes the brainstem and the cerebellum.

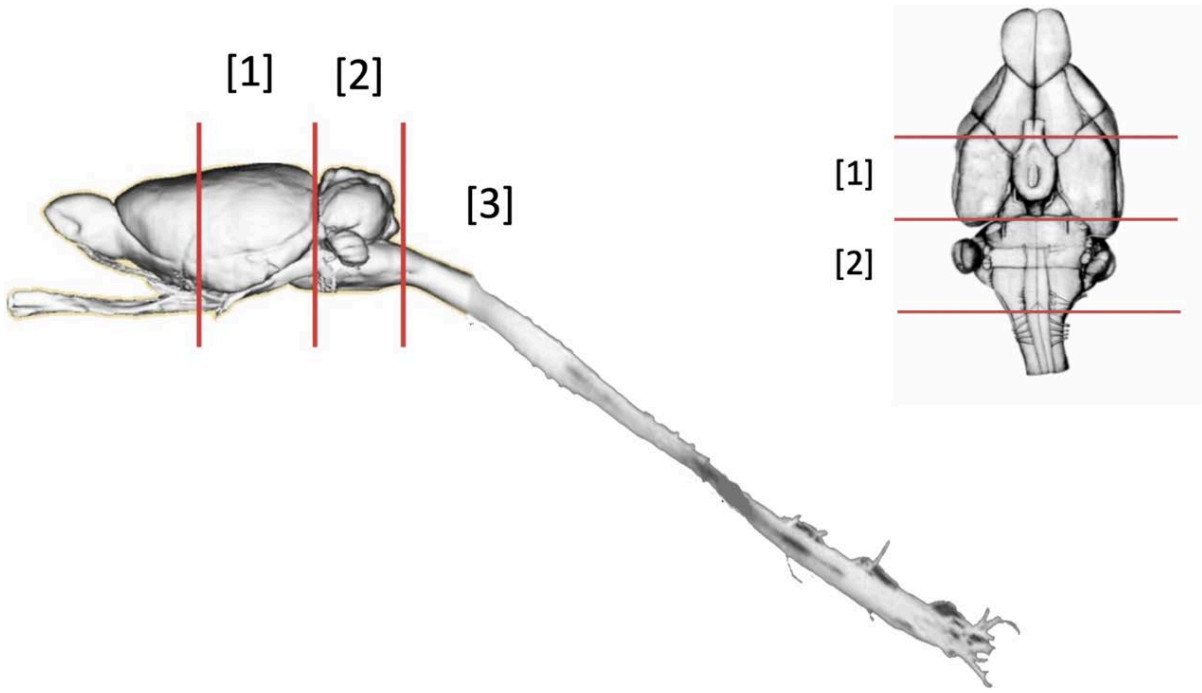


Figure 10. Central Nervous System Regions Collected for ICP-MS Analysis. Schematic representation of the lateral and ventral aspect of the rat brain, showing the CNS regions considered for metal tissue analysis. Region (1) is the cerebrum extending from the midbrain till the optic chiasm, region (2) includes the brainstem and the cerebellum, and region (3) is the spinal cord.

b. ICP-MS Analysis

Inductively Coupled Plasma Mass-Spectrometry (ICP-MS) analysis was used to detect and quantify Gd metal in the collected tissue samples (**Figure 11**). The analysis was performed at the Environmental Core Laboratory (EVL) at the American University of Beirut. For the quantification of gadolinium in the two brain regions, the spinal cords, and peripheral nerves, wet samples were weighed and then digested on a microwave (Anton Paar; Multiwave 3000) using 6 ml of concentrated nitric acid (65% HNO_3; Analar Normapur) and 2 ml of hydrogen peroxide (34.5-36.5% H_2O_2; Sigma-

Aldrich) at approximately 180°C for 30 minutes. The samples were then diluted up to 20 ml. With each digested batch of samples, a blank, a spiked blank, a certified reference material and a matrix spike were used as a quality control. The diluted samples and quality control were measured using an ICP-MS (Agilent 7500ce; Agilent, Waldbronn, Germany). The limit of quantification (LOQ) was 0.013 nmol gadolinium per gram of wet tissue.

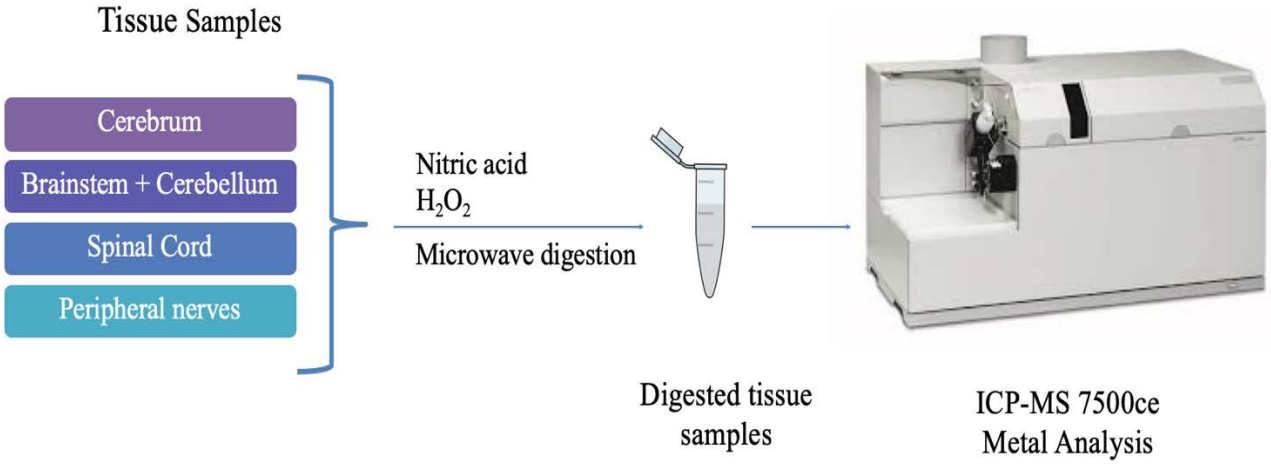


Figure 11. Inductively Coupled Plasma Mass-Spectrometry. Schematic representation showing the sample preparation for Gd metal detection using ICP-MS technique.

C. Statistical Analysis

Assuming normal distribution, the BrdU cell count data and the behavioral data were analyzed using the one-way analysis of variance (ANOVA) test, in which the experimental groups were compared to the controls at each time point. For the ICP-MS data, the unpaired student's *t*-test was used to compare the Gd tissue concentration detected in each dose of the tested contrast agents with the control group. All data were represented as the mean ± the standard error of the mean (SEM). Differences were considered statistically significant at p value < 0.05. The statistical analysis and the plotting of graphs were performed using the Prism 7 GraphPad package (GraphPad software Inc., CA, USA).

CHAPTER III

RESULTS

A. Behavioral Testing: Spontaneous Alternation T-Maze Test

Rats injected with Gadodiamide and Gadoterate-Meglumine showed no statistically significant change on the spontaneous alternation T-maze test performance, as compared to baseline values, control and naïve groups as shown in **Figure 12**. No spatial working memory dysfunction was observed at day 10 and day 20 during the GBCA treatment, and on the long-term one month after the last GBCA injection. However, there was an observed tendency in rats exposed to Gadodiamide to fail the T-maze test at the late timepoint, one month after the last GBCA exposure **(Figure 12)**.

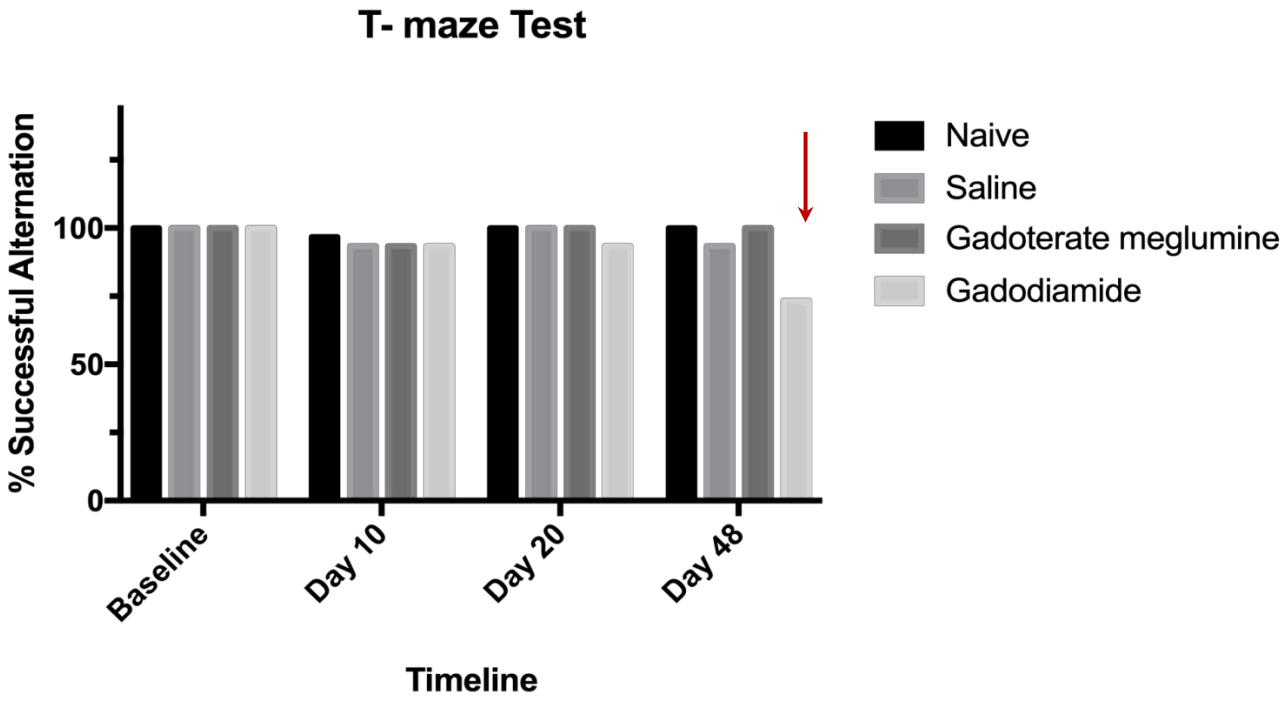


Figure 12. Percentage of successful trials on the spontaneous alternation T-maze test among the different groups at day 10, day 20, and day 48 of the experiment.

B. Effect of Gadolinium Brain Deposition on Cellular Proliferation in the Dentate Gyrus of the Hippocampus

The effect of Gd metal precipitation in the brain on proliferating stem/progenitor cells in the DG of the hippocampus was evaluated using immunohistochemical studies. Intracranial Gd deposits, which were quantified by ICP-MS in this study to be 8.64 ± 0.54 and 8.35 ± 0.96 nmol Gd/g tissue in the cerebrum following the administration of 20 consecutive doses of Gadodiamide and Gadoterate meglumine respectively, did not induce significant alteration on cellular proliferation in the hippocampal DG. As shown in **Figure 13**, the total number of BrdU positive cells in rats exposed to linear and macrocyclic GBCAs and sacrificed 48 hours after the last BrdU injection was comparable to that counted in control and naïve groups. Representative confocal images of the caudal region of the hippocampal dentate gyrus are shown in **Figure 14** and **Figure 15**. Co-localization of the proliferating BrdU + cells with the Hoechst nuclear stain was used to confirm the identity of counted cells.

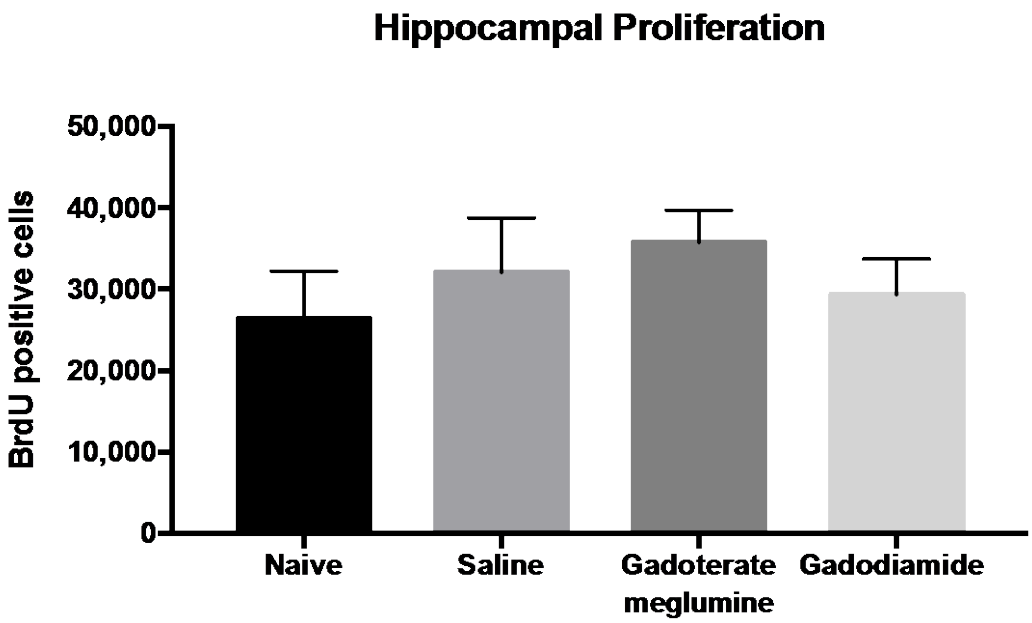


Figure 13: Effect of Gd Brain Deposition on Cellular Proliferation in the DG of the Hippocampus. Quantification of the total number of proliferating $BrdU^+$ cells in the DG of the hippocampus in rats exposed to 20 doses of Gadodiamide and Gadoterate meglumine, and sacrificed 48 hours after the last BrdU injection. Results show no statistically significant change in cellular proliferation in rats exposed to linear and macrocyclic GBCAs, as compared to control and naïve groups. The total cell count represented is the summation of the number of $BrdU^+$ cells counted in the rostral, the intermediate, and the caudal topographic regions of the hippocampus. Data are expressed as mean ± SEM. n=5 rats per group.

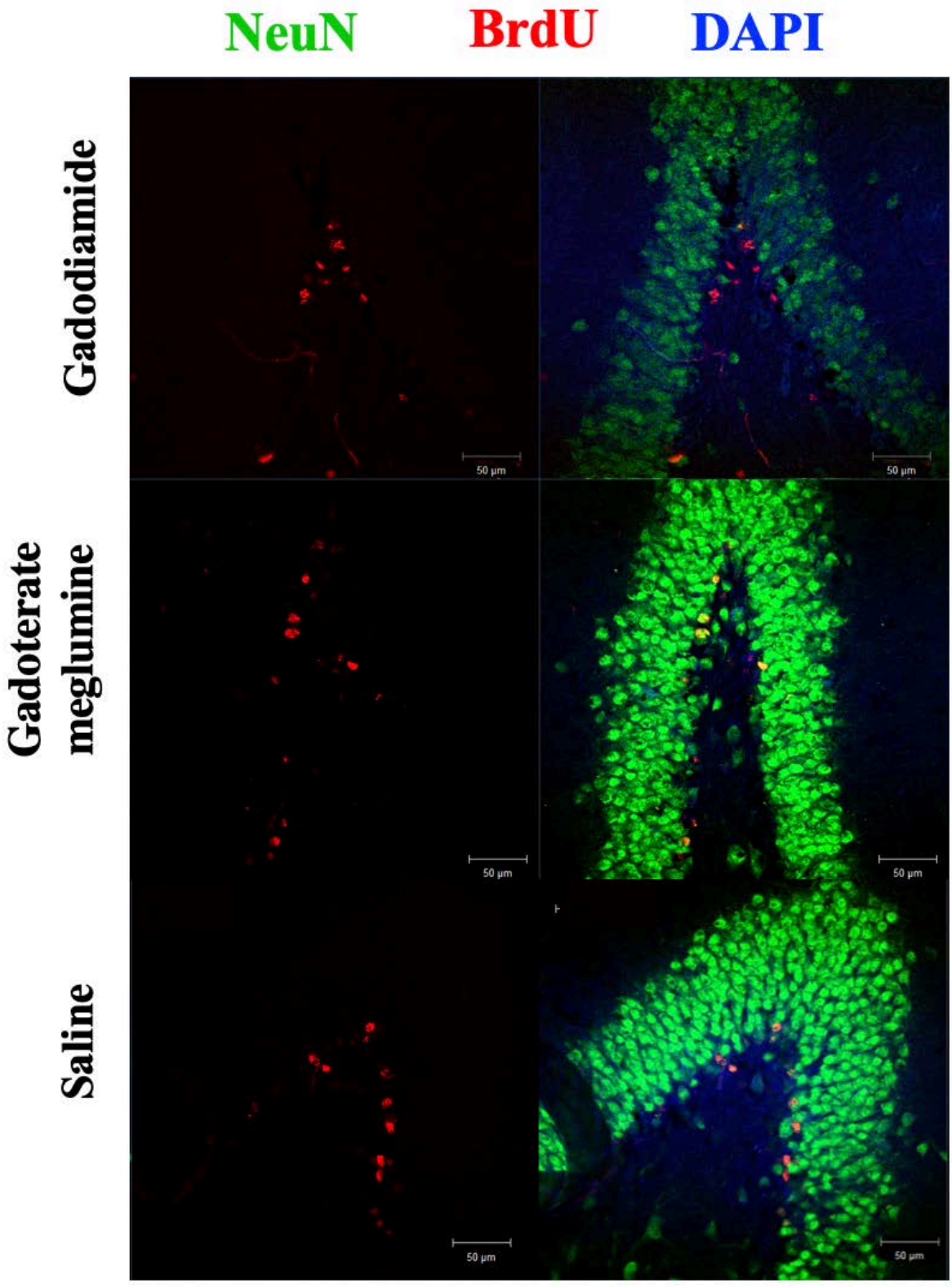


Figure 14: Cellular proliferation in the DG of the hippocampus following exposure to linear and macrocyclic GBCAs. Representative confocal images showing proliferating BrdU positive cells (Red), the mature neuronal marker NeuN (Green), and the Hoechst nuclear stain (Blue) in the caudal region of the hippocampal DG in experimental and control groups sacrificed 48 hours post last BrdU injection. The spatial distribution of stem/progenitor $BrdU^+$ cells can be seen in the sub-granular zone of the DG. The number of $BrdU^+$ cells did not change significantly in animals exposed to multiple injections of Gadodiamide and Gadoterate meglumine as compared to control animals. Tile scan images were taken at 40X oil objective. Scale bar, 50µm.

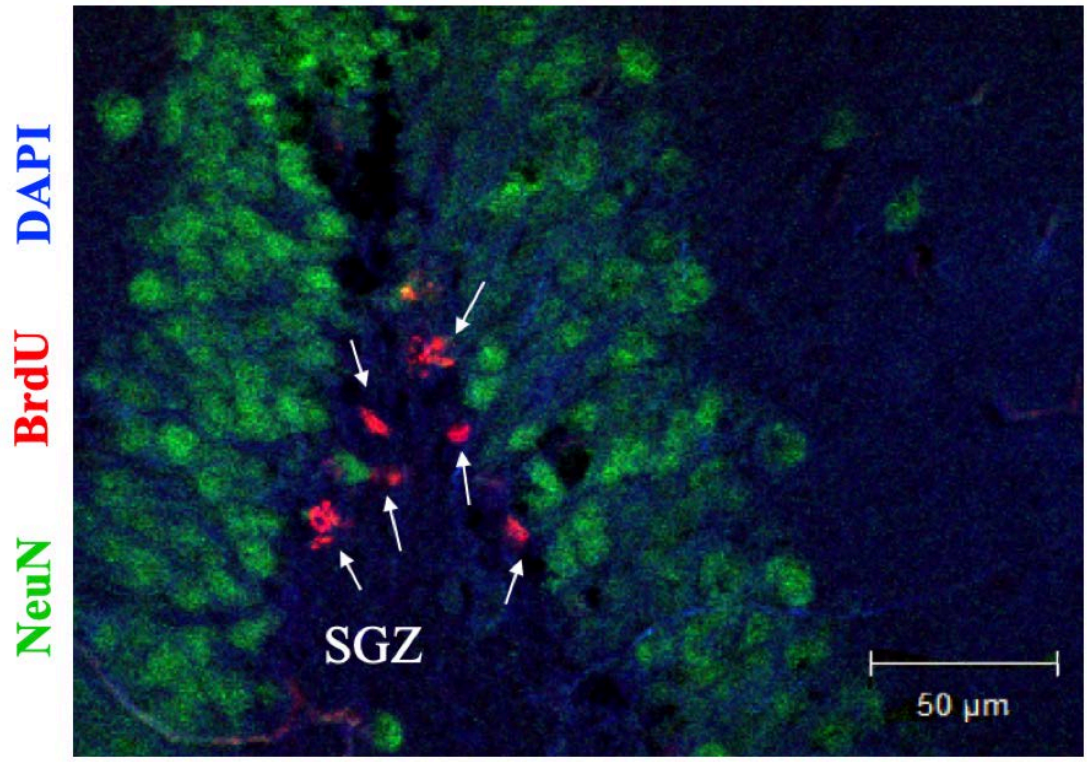


Figure 15: Zoomed confocal image showing the spatial distribution of stem/progenitor $BrdU^{+}$ cells in the sub-granular zone, which is the germinal layer of the DG of the hippocampus. Tile scan taken at 40X oil objective. Scale bar, 50µm. *SGZ: sub-granular zone.*

C. Effect of Gadolinium Brain Deposition on Hippocampal Neurogenesis

The maturation and differentiation of proliferating cells into neurons in the DG in animals exposed to 20 doses of Gadodiamide and Gadoterate meglumine was assessed 29 days after the last BrdU injection. Intracranial Gd deposits did not alter the neuronal differentiation process, maturation, and integration into the GCL of the DG of the hippocampus. As shown in **Figure 16**, there was no significant change in the number of $BrdU^+/NeuN^+$ co-labeled cells in rats exposed to linear and macrocyclic GBCAs as compared to saline and naïve groups. However, there was an observed decreasing trend with the absence of statistical significance in the gadodiamide exposed group. Representative confocal images of the newly born neurons in the DG of the hippocampus are shown in **Figure 17** and **Figure 18**.

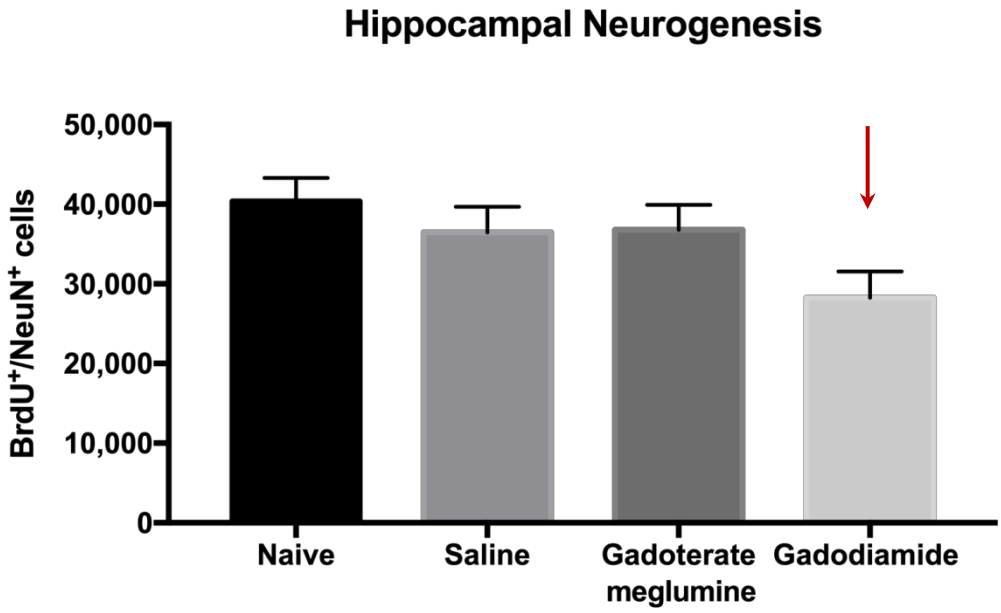


Figure 16: Effect of Gd Brain Deposition on Hippocampal Neurogenesis. Quantification of the total number of double-labeled $BrdU^+/NeuN^+$ newly maturing

neurons in the DG of the hippocampus in rats exposed to 20 doses of Gadodiamide and Gadoterate meglumine, and sacrificed 29 days after the last BrdU injection. Results show no statistically significant change in the hippocampal neurogenesis of rats exposed to linear and macrocyclic GBCAs, as compared to control and naïve groups. The total cell count represented is the summation of the number of $BrdU^{+}/NeuN^{+}/Dapi^{+}$ cells counted in the rostral, the intermediate, and the caudal topographic regions of the hippocampus. Data are expressed as mean ± SEM. n=5 per group.

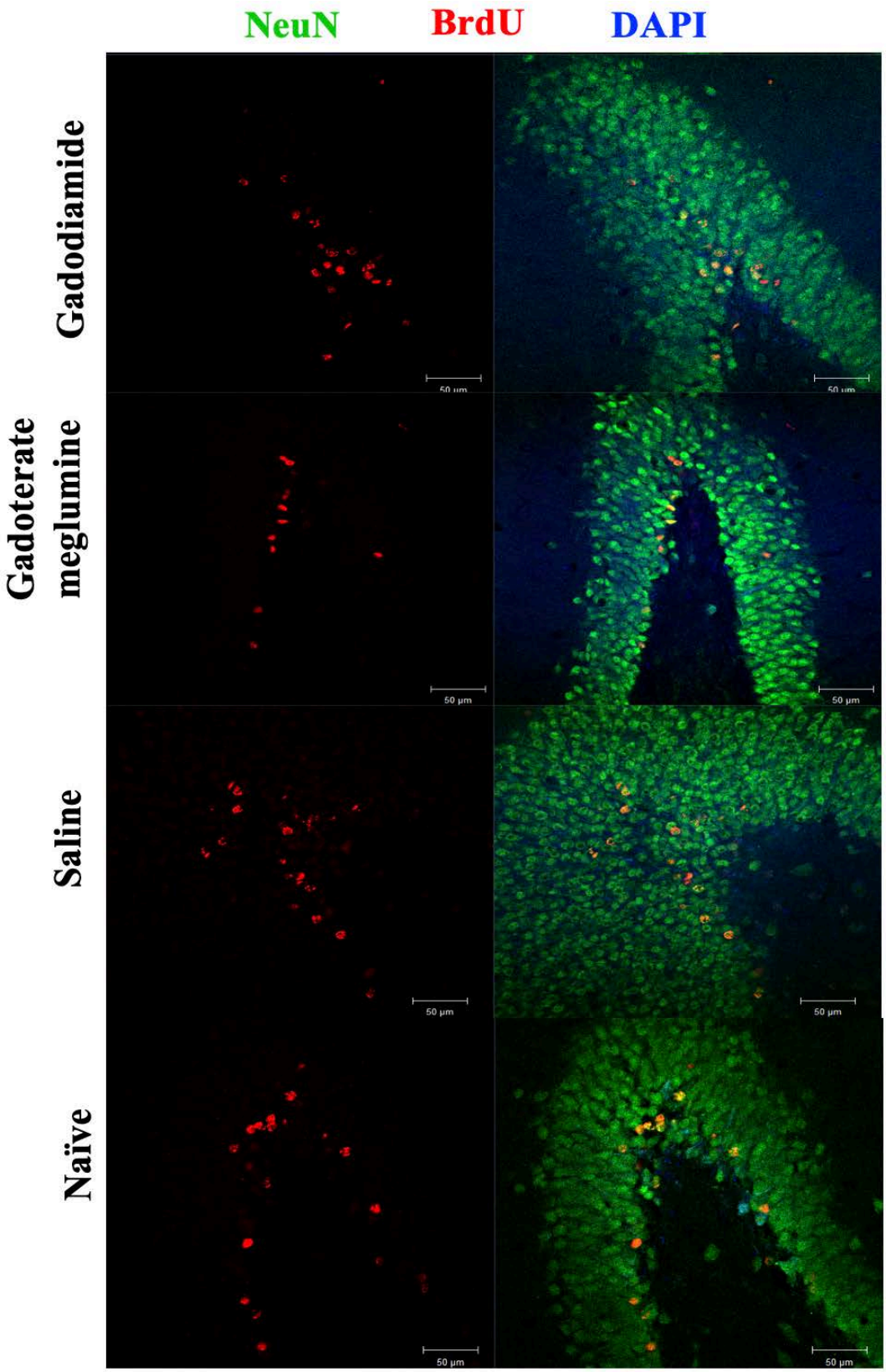


Figure 17: Hippocampal Neurogenesis following Intracranial Gd Deposition. Confocal images showing $BrdU^{+}/NeuN^{+}$ co-labeled cells in the DG of the hippocampus in experimental and control groups sacrificed 29 days post last BrdU injection. The Basal level of neurogenesis in the DG in naïve and control groups did not significantly differ from animals exposed to multiple Gadodiamide and Gadoterate meglumine injections. Newly born neurons can be seen integrated into the GCL of the DG of the hippocampus. Tile-scan images taken at 40X oil objective. Scale bar, 50µm.

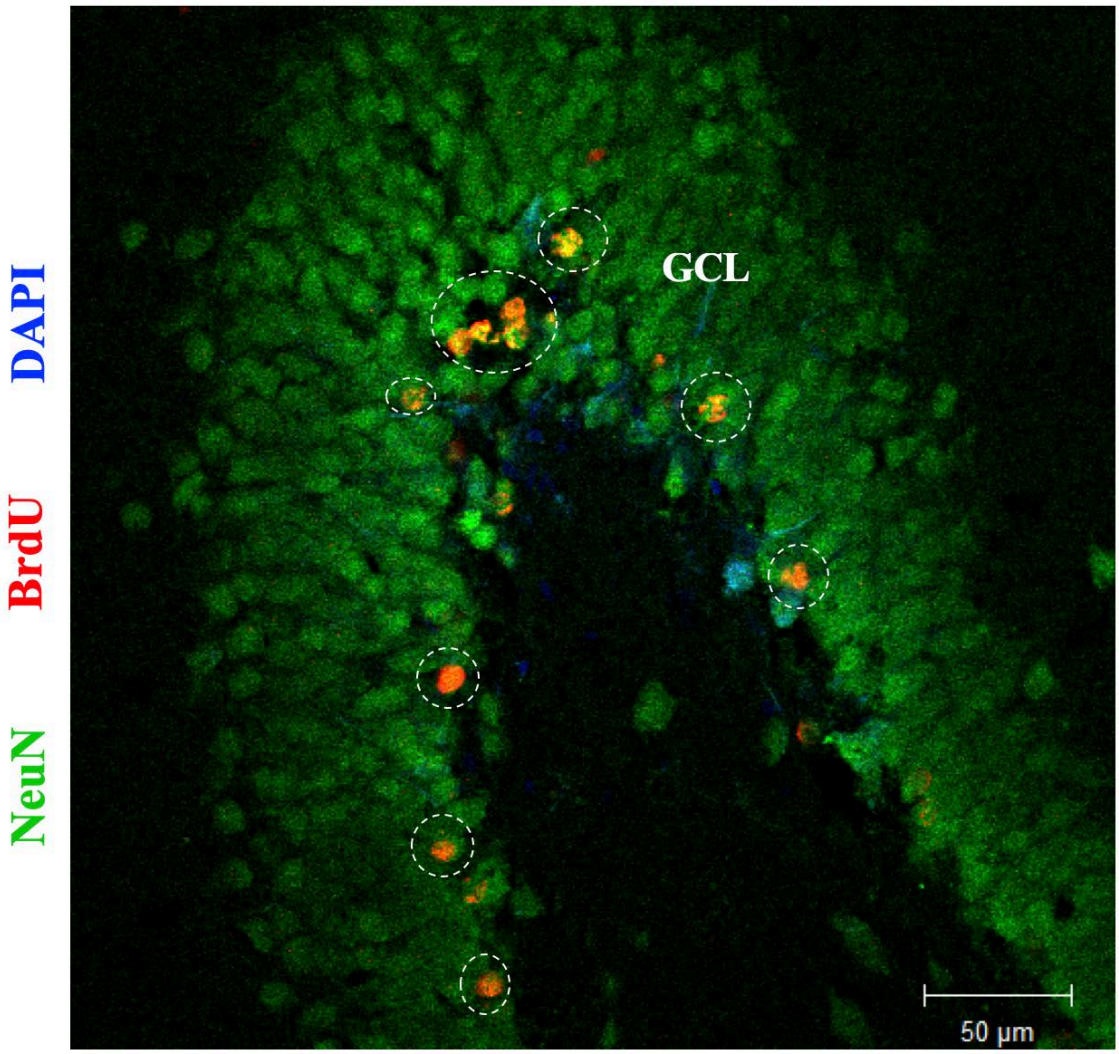


Figure 18: Zoomed confocal image showing the integration of newly born neurons into the granular cell layer of the DG of the hippocampus 29 days after the last BrdU injection. Tile scan images taken at 40X oil objective. Scale bar, 50µm. *GCL: granular cell layer.*

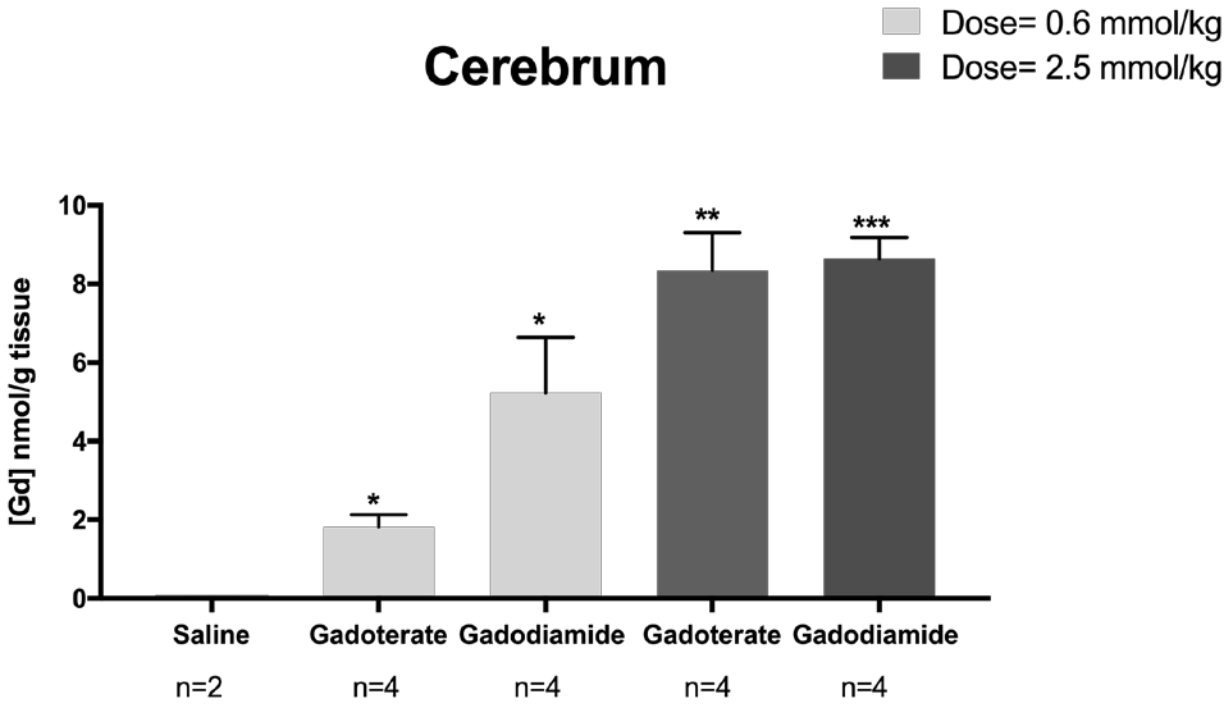
D. Determination of Gd Concentration in the Central and Peripheral Nervous System using ICP-MS

All tested GBCAs resulted in significant Gd metal deposition in the central and peripheral nervous tissue, as compared to the saline control group **(Figure 19 A-D).** For the high dosage groups, the average detected total Gd concentrations (in nmol Gd per g of tissue) for Gadodiamide and Gadoterate meglumine, respectively, were as follows: 8.64 ± 0.54 and 8.35 ± 0.96 nmol/g in the cerebrum, 6.87 ± 0.54 and 5.19 ± 2.4 nmol/g in the brainstem and cerebellum, 47.6 ± 6.74 and 5.02 ± 0.59 nmol/g in the spinal cord, and 103 ± 6.48 and 1.04 ± 0.07 nmol/g in the peripheral nerves. Such metal concentrations were significantly higher than the saline control group with 0.019 ± 0.006 nmol/g in the cerebrum, 0.056 ± 0.043 nmol/g in the brainstem and cerebellum, 0.031 ± 0.017 nmol/g in the spinal cord, and 0.18 ± 0.03 nmol/g in peripheral nerves. The mean detected residual Gd concentration in the spinal cord was approximately 9.4-folds higher for linear than for the macrocyclic GBCA **(Figure 19C).** Whereas for the peripheral nerves, exposure to Gadodiamide resulted in a significantly high metal deposition in the sciatic and trigeminal nerves as compared to Gadoterate meglumine that resulted in a much lower nerve tissue deposition **(Figure 19D)**.

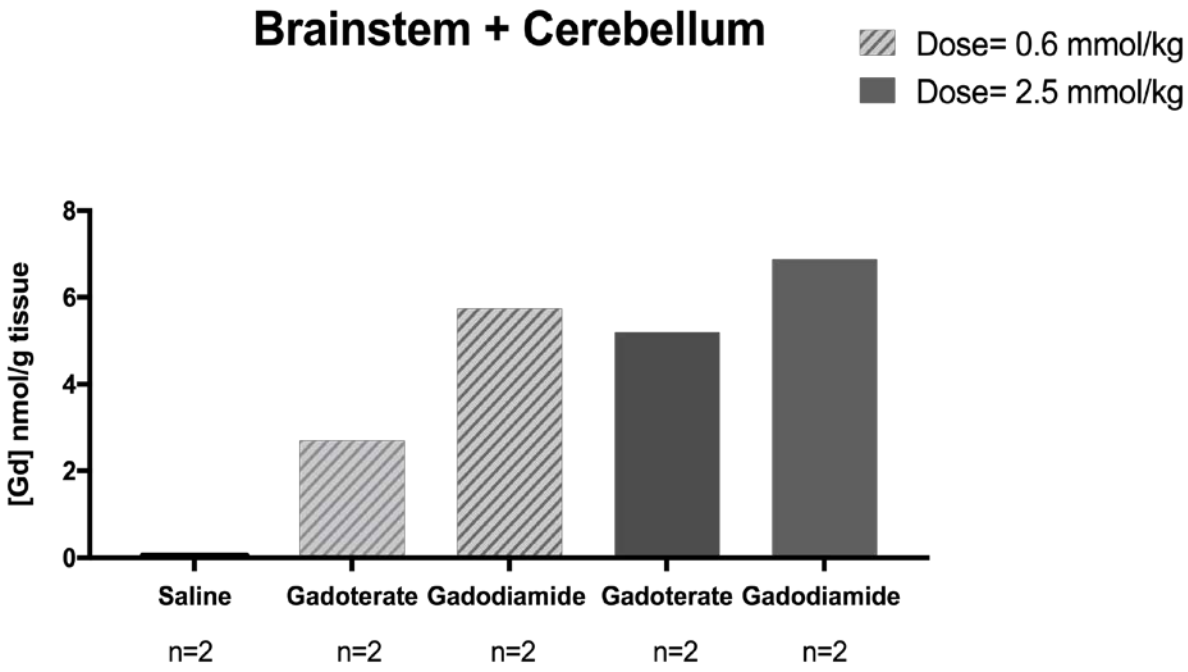
Furthermore, for the low dosage groups (0.6 mmol/kg: dose equivalent to the clinically administered human dose), the average detected Gd tissue concentrations were 5.24 ± 1.41 nmol/g in the cerebral region, 5.74 ± 1.15 nmol/g in the brainstem and cerebellum, and 14.15 ± 3.10 nmol/g in the spinal cord for rats exposed to Gadodiamide. Whereas for Gadoterate meglumine, the Gd tissue concentrations were 1.83 ± 0.3 nmol/g in the cerebrum, 2.7 ± 1.30 nmol/g in the brainstem and cerebellum, and 2.03 ± 0.48 nmol/g in the spinal cord . Accordingly, the Gd concentration in the

spinal cord following repeated linear GBCA administration was approximately 7-folds higher than after repeated exposure to the macrocyclic GBCA **(Figure 19C)**. Moreover, for the cerebrum, the brainstem and cerebellum structures, linear agents lead to approximately 2-fold and 3-folds increase in tissue deposition than macrocyclic GBCAs respectively **(Figure 19A and 19B)**.

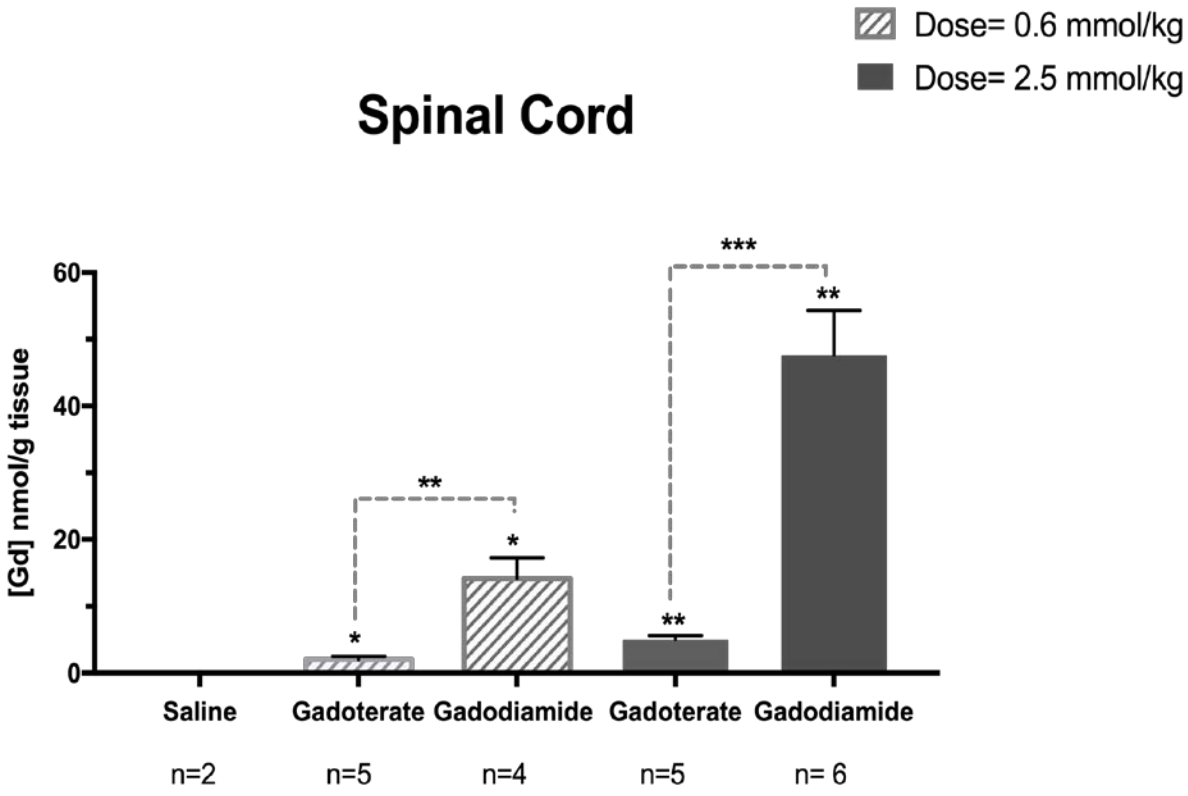
A.



B.


Brainstem + Cerebellum
Dose= 0.6 mmol/kg
Dose= 2.5 mmol/kg
[Gd] nmol/g tissue
8
6
4
2
0
Saline
Gadoterate
Gadodiamide
Gadoterate
Gadodiamide
n=2
n=2
n=2
n=2
n=2

C.


Dose= 0.6 mmol/kg
Dose= 2.5 mmol/kg
Spinal Cord
[Gd] nmol/g tissue
60
40
20
0
***
**
**
*
*
Saline
Gadoterate
Gadodiamide
Gadoterate
Gadodiamide
n=2
n=5
n=4
n=5
n= 6

D.

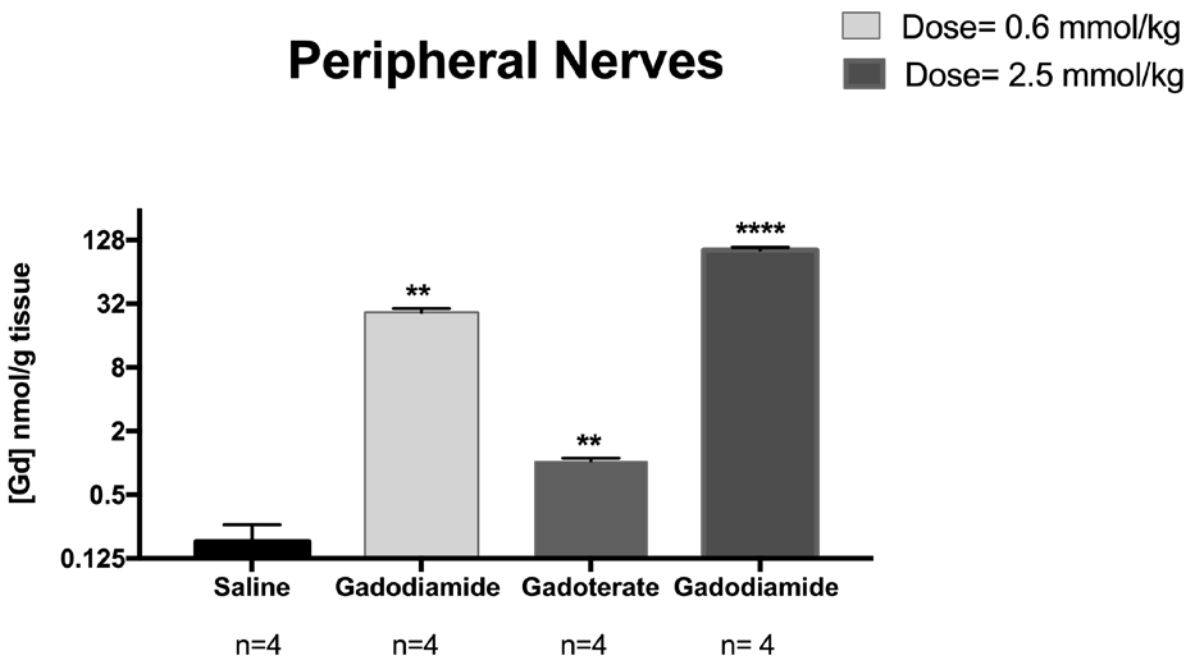


Figure 19. Quantification of Gd metal using ICP-MS in the Central and Peripheral Nervous Tissue. Two doses of Gadodiamide and Gadoterate meglumine were used: the supra-clinical dose (2.5 mmol/kg), and a dose equivalent to the human dose (0.6 mmol/kg). [Gd] following the administration of 20 daily injections was measured in the following tissues: **(A)** the Cerebral region extending from the optic chiasm to the midbrain, **(B)** the Brainstem and Cerebellum, **(C)** the Spinal Cord, and **(D)** the Sciatic and Trigeminal peripheral nerves. In all analyzed regions, Gadodiamide resulted in higher Gd tissue concentration than the macrocyclic agent Gadoterate meglumine. [Gd] is expressed in nanomole Gd/gram of tissue. The number of animals (n) tested in the different groups are indicated below the graph. Data are expressed as mean ± SEM. Significance with reference to the control group is noted by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CHAPTER IV

DISCUSSION

The overall results of this exploratory study indicate that Gadolinium retention in the brain following the exposure to repeated doses of Gd-based MRI contrast agents does not significantly affect the dynamic process of hippocampal neurogenesis or alter the spatial working memory performance in young rats. Nevertheless, a non-statistically significant decrease in the rate of hippocampal neurogenesis and behavioral outcomes was clearly observed in the gadodiamide-exposed group one month after the last GBCA injection. This decreasing trend needs to be further investigated. Furthermore, our study provides the first evidence for Gd metal deposition in the spinal cord and peripheral nerves following the exposure to the linear and macrocyclic GBCAs, Gadodiamide (Omniscan®) and Gadoterate meglumine (Dotarem®) respectively.

In accordance with previously published animal studies, the current study used an extended dosing regimen of GBCA administration in order to create a rodent model of Gd brain deposition. According to the literature, multiple serial injections are needed to induce Gd deposits in the rodent brain in a similar manner to that observed in humans clinically, replicating the same brain MRI signal intensity changes (Jost et al., 2016; Kartamihardja et al., 2016; Robert et al., 2015; Robert et al., 2016). This is due to species differences, as the excretion half-life of GBCAs in rats is 20 minutes, leading to an approximately five times faster renal elimination than in humans that have a 1.5 - 2 hours half-life (Jost et al., 2018; Oksendal & Hals, 1993; Robert et al., 2016). Furthermore, the Gd tissue quantification performed in this study followed two dosing regimens: the low dose (0.6 mmol/kg) which is equivalent to the clinically administered

human dose after the adjustment for species differences (FDA Center for Drug Evaluation and Research, 2005), and the high dose 2.5 mmol/kg, which represents the optimal dose required for brain MRI enhancement following intraperitoneal GBCA administration in rodents (Portnoy, Bishop, Dazai, Spring, & Henkelman, 2008). Even though 2.5 mmol/kg is a high dose, it is considered significantly lower than the median lethal dose (LD_{50}) for the tested GBCAs in rodents (Omniscan-LD_{50}= 30 mmol/kg and Dotarem-LD_{50}= 11 mmol/kg) (Cacheris, Quay, & Rocklage, 1990; Harpur et al., 1993; Meyer, Schaefer, & Doucet, 1990; Oksendal & Hals, 1993).

The lack of significant effects of GBCAs on working memory function and neurogenesis rate following intracranial Gd deposition in the developing brain of young rats is inconsistent with the clinical observation indicated by Miller et al. (Miller et al., 2015). Miller et al. reported a case of a pediatric patient who received a total of 35 linear GBCA injections between the age of 8 and 20 years old, in which signal hyperintensity was evident in the patient's GP, DN, thalamus and pons on un-enhanced MRI. Neuropsychological testing revealed an impairment in the patient's visual and working memory performance, in executive cognitive functions such as planning and organization, in addition to weak mathematical and reading abilities. However, no definite strong association between Gd intracranial deposition and memory dysfunction was concluded in the reported case, since the patient had a history of a brain tumor, radiation and chemotherapy (Miller et al., 2015). However, our behavioral and immunohistochemical results are consistent and further support the recent population-based clinical study by Mcdonald et al. which reported no association between GBCA exposure and neurocognitive dysfunction (RSNA, 2017). Our preclinical study revealed that Gd metal existence in the neuronal environment is not an independent factor

significantly affecting hippocampal neurogenesis or working memory function in the brain. However, due to the observed decreasing trend in the number of newly maturing neurons and behavioral outcomes in animals exposed to the linear GBCA, Gadodiamide, at the late time point, a power analysis was conducted to examine whether Gadodiamide exposure could lead to a statistical significant effect on hippocampal related functions and neurogenesis. The analysis revealed that 10 animals/group is the minimum sample size required to achieve statistical significance. In addition, further behavioral studies are needed to confirm the lack of effect of linear and macrocyclic GBCAs on cognitive functions. Even though the T-maze test is highly sensitive for detecting hippocampal dysfunction without inducing stress to the tested animals (Deacon & Rawlins, 2006; Lalonde, 2002), testing different aspects of learning and memory is highly warranted.

Gd tissue concentrations detected in the different brain regions in this study following both dosing regimens are consistent and comparable with previously published animal experiments (Frenzel et al., 2017; R. J. McDonald, J. S. McDonald, D. Dai, et al., 2017; Robert et al., 2015; Robert et al., 2016; Smith et al., 2017), with the linear GBCA gadodiamide leading to higher Gd retention than the macrocyclic agent Gadoterate meglumine. Nevertheless, the slight increase in the ICP-MS measurements in the brain in our study might be due to several reasons, including the use of the *i.p.* route of injection that might result in higher tissue deposition as suggested by Neidl van Gorkom et al. (Neidl van Gorkom, Mohamed, Labada, & Langer, 2015; Neidl van Gorkom et al., 2012). Hypothetically, the slow and delayed absorption of the administered GBCAs from the peritoneal cavity might result in an extended exposure to the injected chemical substances. Moreover, the majority of previously published

animal studies followed 4-5 GBCA injections per week regimen, whereas in this study a daily consecutive GBCA administration protocol was followed contributing to our higher ICP-MS data. We have found that the use of the *i.p.* route for GBCAs administration instead of the intravenous, is convenient and practical for drug administration to rodents over an extended long period of time (20 days). The *i.p.* route was chosen in order to avoid inducing daily stress, pain, discomfort, restrain, vein sclerosis or injury to the tested animals, and to avoid administering inhalation anesthesia which are all considered major confounding variables capable of significantly affecting the process of hippocampal neurogenesis and cognitive behavioral outcomes to be studied.

Furthermore, our exploratory study shed the light and revealed a significant amount of retained Gd in the spinal cord, which is alarming. We speculate that the mechanism of Gd deposition in the spinal cord is analogous to that of the brain, possibly through the glymphatic system pathway, in which GBCAs distribute via the CSF in the central canal then enter the spinal cord parenchyma through the perivascular spaces surrounding penetrating vessels. While Gadoterate meglumine resulted in reduced tissue retention in the spinal cord and peripheral nerves than Gadodiamide, the safety of GBCAs should be further evaluated. More research is needed to assess the impact of such deposition on sensory and motor neuronal activities. Gd deposition in the spinal cord and peripheral nerves might possibly contribute to the pathophysiology of the sensory symptoms and burning pain in the torso and extremities described by some patients following GBCAs administrations in several reports (Burke et al., 2016; Semelka, Commander, et al., 2016; Semelka, Ramalho, Vakharia, et al., 2016). Eventually, attention must be driven to the long-term effect of such CNS and PNS metal

deposition especially on children and adults with medical conditions such as CNS tumors, spinal cord pathologies, or multiple sclerosis that require multiple CE-MRIs. Future research shall focus on localizing and quantifying the Gd metal deposits in different regions of the spinal cord, and on assessing any resulting histopathological or molecular changes. Moreover, future clinical imaging studies shall investigate if there are any changes in the signal intensity of the spinal cord on unenhanced T1-weighted MRIs in patients exposed to multiple GBCA doses.

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