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Infusion-based manganese-enhanced MRI: a new imaging technique to visualize the mouse brain

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Abstract

Manganese-enhanced magnetic resonance imaging is a technique that employs the divalent ion of the paramagnetic metal manganese (Mn^{2+}) as an effective contrast agent to visualize, in vivo, the mammalian brain. As total achievable contrast is directly proportional to the net amount of Mn^{2+} accumulated in the brain, there is a great interest in optimizing administration protocols to increase the effective delivery of Mn^{2+} to the brain while avoiding the toxic effects of Mn^{2+} overexposure. In this study, we investigated outcomes following continuous slow systemic infusion of manganese chloride ($MnCl_2$) into the mouse via mini-osmotic pump administration. The effects of increasing fractionated rates of Mn^{2+} infusion on signal enhancement in regions of the brain were analyzed in a three-treatment study. We acquired whole-brain 3-D T1-weighted images and performed region of interest quantitative analysis to compare mean normalized signal in Mn^{2+} treatments spanning 3, 7, or 14 days of infusion (rates of 1, 0.5, and 0.25 $\mu L/h$, respectively). Evidence of Mn^{2+} transport at the conclusion of each infusion treatment was observed throughout the brains of normally behaving mice. Regions of particular Mn^{2+} accumulation include the olfactory bulbs, cortex, infralimbic cortex, habenula, thalamus, hippocampal formation, amygdala, hypothalamus, inferior colliculus, and cerebellum. Signals measured at the completion of each infusion treatment indicate comparable means for all examined fractionated rates of Mn^{2+} infusion. In this current study, we achieved a significantly higher dose of Mn^{2+} (180 mg/kg) than that employed in previous studies without any observable toxic effects on animal physiology or behavior.

Keywords

MRI; Manganese; Oxytocin; Osmotic pump; Infusion

Introduction

Magnetic resonance imaging (MRI) has emerged as a major non-invasive technique to visualize the brain in both animal and clinical studies. Harnessing the physical properties and interactions of some atomic nuclei via the application of an external magnetic field, MRI provides structural and functional data that can be examined in vivo longitudinally (Budinger et al. 1999; Farrall 2006). Such uses have promoted MRI as a popular method to

observe anatomical and functional characteristics of the brain; particularly, regarding transgenic animal studies of disease.

Despite MRI's ability to provide images with spatial resolutions of <0.2 mm, there has been much recent interest in contrast agents to enhance the observation of morphological structures and functional activity in the brain (Budinger et al. 1999). These agents include paramagnetic metals such as gadolinium and manganese (Caravan et al. 1999; Krause et al. 2002). Manganese-enhanced MRI (MEMRI) is becoming an increasingly popular means of imaging animals due to the ability of Mn^{2+} to enter neurons via voltage-gated calcium ion channels. Upon depolarization, these voltage-dependent calcium channels permit the entry of Mn^{2+} due to the ion's similar ionic radius to that of Ca^{2+} (Lin and Koretsky 1997; Kuo et al. 2006; Yu et al. 2005). Thus, Mn^{2+} uptake indicates direct activity in the brain, which is observed in MRI as enhanced signal intensity proportionate to the frequency of neuron depolarization. This method of activity-dependent imaging differs from traditional methods of functional MRI that interpret activation as a function of hemodynamic fluctuations in oxygen concentration. The limitations of blood oxygenation level-dependent (BOLD) fMRI are apparent in its requirement that animals be immobilized completely during stimulation and functional activity acquisition. Subsequently, extended periods of physical restraint in a low-temperature, loud magnet bore preclude the expression of many natural behaviors exhibited typically in the animal's normal environment (Ferris et al. 2008).

The combination of manganese's ionic properties in biological systems, its physical ability to shorten T1-relaxation times (thus enhancing signal), and its slow rate of clearance from rodent brains (half life >1.5 months) has proven MEMRI to be an invaluable technique to delineate brain structure, function, and neuronal connectivity (Aoki et al. 2004; Pautler et al. 2003; Takeda et al. 1995). Manganese's chief disadvantage, however, is its cellular toxicity, which can lead to neurological deficits and organ failure when administered at high doses (Crossgrove and Zheng 2004; Malecki 2001; Aschner and Aschner 1991). As a result, attempts to increase Mn^{2+} concentration per dose to further enhance MR signal have been inhibited. Such an obstacle is critical to overcome in functional studies that require physiologically healthy and normally behaving animals. The use of several fractionated dose administrations of manganese to improve image contrast has been demonstrated to minimize toxicity and produce greater signal enhancement at higher total doses (Bock et al. 2008; Grünecker et al. 2009). However, multiple injections conducted intraperitoneally in awake animals or intravenously in anesthetized animals are too intrusive for some behavioral studies, such as those focused on anxiety-like behaviors. A recent study examined the use of an osmotic pump to achieve continuous slow, systemic release of Mn^{2+} in rats (Eschenko et al. 2010). These authors used intra-abdominal osmotic pumps to deliver a total of 80 mg/kg of $MnCl_2$ over 7 days and found no decreases in motor performance. In contrast, an equivalent single bolus dose administered systemically was found to reduce wheel running, as well as food intake and body weight. The method of manganese infusion via osmotic pump implantation is thus less disruptive to the animal's mobility and optimal in studies on animal behavior. Nevertheless, systematic analysis of the relationship between the rate of continuous systemic manganese infusion and signal intensity in the brain has not yet been explored. This relationship is important considering the diversity of behavioral tasks employed commonly on rodent models that range in frequency and duration.

In this study, we employed MRI to examine the effects of the rate of manganese administration via osmotic pump infusion on signal intensity in the brains of a transgenic line of oxytocin receptor-deficient (*Oxtr* KO) adult male mice (C57BL/6J background) (Lee et al. 2008) and in a control group of C57BL/6J age-matched male mice. The importance of oxytocin in reproduction, learning, memory, stress response, and social behaviors has fostered much interest in the gene knockout model of the peptide's only receptor (Lee et al.

2009). MEMRI's ability to visualize neuronal function in the brain is useful for examining basal activity levels in areas of particular *Oxtr* density (i.e., hypothalamus, olfactory system, amygdala, and hippocampus) in *Oxtr* KO versus controls. Utilizing whole-brain 3-D T1-weighted imaging in combination with systemic manganese administration via implantation of a mini-osmotic pump apparatus, we had three chief aims: (1) to look for differences in signal intensity among three different rates of MnCl_2 infusion (1, 0.5, and 0.25 $\mu\text{L/h}$; same final total amounts of MnCl_2) performed over 3, 7, and 14 days, respectively; (2) to observe obvious signs of toxicity from the MnCl_2 administration and (3) to compare Mn^{2+} accumulation in the brains of normally behaving *Oxtr* KO and C57BL/6J control mice.

Materials and methods

Subjects and Mn^{2+} administration

Three cohorts of male *Oxtr* KO mice (C57BL/6J background) and control male C57BL/6J age-matched adult mice (~22–30 g) were evaluated ($n = 4$ per cohort) in this study. They were generated and genotyped as previously described (Lee et al. 2008). As shown in Fig. 1, each cohort was assigned to one of three Mn^{2+} infusion treatments: 3-day (1.0 $\mu\text{L/h}$), 7-day (0.5 $\mu\text{L/h}$), or 14-day (0.25 $\mu\text{L/h}$). Each treatment provided a cumulative total dose of 180 mg/kg of MnCl_2 (~3.96–5.4 mg/mouse) ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, Sigma-Aldrich, St. Louis, MO, USA). An additional cohort of *Oxtr* KO and wildtype age-matched adult male mice treated with the 3-day Mn^{2+} infusion (total dose of 180 mg/kg) was also examined in this study ($n = 7$ WT, $n = 5$ KO). In these animals, we conducted daily measurements of weight ($n = 6$). All MnCl_2 solutions were prepared in 400 mM bicine buffer, pH of 7.4 (adjusted with 1 M NaOH). Continuous slow systemic infusion of MnCl_2 was achieved through subcutaneous implantation of mini-osmotic pumps (ALZET, DURECT Corporation, Cupertino, CA, USA) on the midline of the mid-lower back of the mice. Osmotic pumps utilized in this study included the 1003D (3-day), 1007D (7-day), and 1002 (14-day) models, all of uniform mass and size (0.4 g, 1.5 cm in length, 0.6 cm in diameter). The pumps contained stainless steel flow moderators and were thus removed prior to MR scanning. We performed all surgical operations on mice anesthetized with 2% isoflurane. Mice were individually housed and provided food and water ad libitum throughout the duration of the infusion treatments. The physical health and activity of all treated animals were assessed daily by animal care staff and veterinarians of the National Institute of Mental Health (NIMH) small animal facility. The NIMH Animal Care and Use Committee (ACUC) approved all animals and methods utilized in this study.

T1-weighted MR acquisition

For each of the Mn^{2+} infusion treatments, we performed T1-weighted MRI at two time points: pre-infusion and post-infusion. Whole-brain 3-D images were acquired using a T1-weighted gradient echo sequence in a 7 T 21-cm horizontal scanner (Bruker BioSpin, Billerica, MA). The MR acquisition protocol consisted of 1.5-h scans collected over eight averages (echo time = 3.5 ms, repetition time = 30 ms, isotropic spatial resolution = 100 μm , field of view = $1.56 \times 1.5 \times 1.2$ cm, image matrix = $256 \times 150 \times 120$). In the additional cohort of *Oxtr* KO and WT mice ($n = 7$ WT, $n = 5$ KO) treated with a 3-day Mn^{2+} infusion, MR acquisition protocol consisted of 2-h scans collected over 40 averages (echo time = 3.5 ms, repetition time = 30 ms, spatial resolution = $75 \times 75 \times 150$ μm , field of view = $1.4 \times 1.4 \times 0.48$ cm, image matrix = $192 \times 186 \times 32$).

All animals were secured in a stereotaxic holder and mounted in a 72-mm volume (transmit)/25-mm surface (receive) radio frequency coil ensemble. Mice were maintained under anesthesia (2% isoflurane in a mixture of oxygen, medical air, and nitrogen) and core body temperature was regulated at 37°C using a circulating water pad and monitored via a

rectal temperature probe. A pressure transducer (SA Instruments, Inc., NY, USA) was placed on the dorsal side of the mice to monitor breathing rate, which was maintained at 60 ± 10 breaths/min. A copper sulfate fiduciary marker was placed adjacent to the mouse head in each scan.

Data analysis

We conducted region of interest (ROI) analysis on all acquired pre- and post-infusion images to assess for differences in Mn^{2+} accumulation by treatment group (3-, 7-, and 14-day) and genotype (*Oxtr* KO vs. controls). Regions of Mn^{2+} accumulation examined included the bilateral olfactory bulbs, hippocampal formation, and amygdalae. T1-weighted MR data were analyzed with the ImageJ software (Rasband 1997–2011). ROI's in coronal brain slices were identified and delineated using the Allen Institute Mouse Brain Reference Atlas (Seattle, WA, USA). Raw signal measurements analyzed in each ROI included signal intensity and area in arbitrary units. Signal activity in a ROI was interpreted as a function of Mn^{2+} accumulation—calculated as the product of mean signal intensity and area. All ROI mean signal intensities were thresholded at a constant level and normalized to that of the fiduciary marker included in each scan. We employed the non-parametric independent samples *t* test (Wilcoxon–Mann–Whitney test) and repeated measures analysis of variance (Kruskal–Wallis test) in SPSS Statistics 17.0 (IBM, Somers, NY, USA) to assess the significance between normalized mean signals in each experimental group. Significance was set at $P < 0.05$ for each statistical test.

Results

Mn^{2+} accumulates throughout the whole-brain and concentrates preferentially in active regions

The signal enhancement examined in three mutually perpendicular planes in T1-weighted images of *Oxtr* KO and control mice demonstrate the extent to which Mn^{2+} distributes throughout the mouse brain and the anatomical structures in which Mn^{2+} accumulates. Figure 2 displays representative slice images drawn from a control C57BL/6J adult male mouse after a treatment of 180 mg/kg MnCl_2 infused systemically at a rate of 1 $\mu\text{L/h}$ over 3 days. Signal enhancement is observed in the posterior to anterior areas of the brain as indicated in coronal slice images (Fig. 2a). Alternate axial views of the brain also indicate manganese distribution in slice images left to right and ventral to dorsal in the sagittal (Fig. 2b) and horizontal (Fig. 2c) planes, respectively.

Figure 3 presents enlarged slice images from a control adult male mouse after a treatment of 180 mg/kg MnCl_2 infused systemically at a rate of 1 $\mu\text{L/h}$ over 3 days. Representative horizontal (Fig. 3a), sagittal (Fig. 3b), and coronal (Fig. 3c) views demonstrate abundant accumulation of Mn^{2+} in various structures of the brain. Such regions include the olfactory bulbs, areas of the cortex (i.e., infralimbic cortex, motor cortex, and visual cortex), habenula, hippocampal formation (CA1, CA2, CA3, and dentate gyrus), cerebellum, inferior colliculus, amygdala, pituitary, thalamus, and hypothalamus. It should be noted that many of these cited regions of high- Mn^{2+} accumulation are key components in sensory and limbic systems regulating olfaction, emotion, learning, and memory (Isaacson 2001; Mega et al. 1997).

Equal doses of Mn^{2+} infused at different rates produce comparable signals

To test differences in image quality of pre- and post-infusion of the contrast agent, we analyzed mean signals measured bilaterally from the hippocampal formation, olfactory bulbs, and amygdalae (Fig. 4). We found normalized mean signals in post-infusion images to be significantly greater than that of pre-infusion images ($P < 0.001$) (Fig. 5) in both *Oxtr*

KO and control animals. Despite an apparent trend of greater signal corresponding to higher rates of infusion, non-parametric statistical analysis of signal means did not reveal significant differences in the bilateral olfactory bulbs, hippocampal formations, or amygdalae (or the whole-brain, data not shown) in the 3-, 7-, 14-day infusion treatments (equal cumulative doses of 180 mg/kg, significance levels set at $P < 0.05$; Fig. 5). Qualitative observations of signal intensity are presented from sagittal (Fig. 6a), coronal (Fig. 6b), and horizontal (Fig. 6c) perspectives in representative slice images from each infusion treatment. No significant differences were observed between the *Oxtr* KO and control mice in mean signals measured bilaterally from the olfactory bulbs, hippocampal formation, and amygdalae (data not shown, significance levels set at $P < 0.05$). Repeated analysis conducted on an additional cohort of 3-day Mn^{2+} infused animals also found no significant differences in normalized mean signal measured from the hippocampal formation and whole amygdalae between *Oxtr* KO ($n = 5$) and WT ($n = 7$) adult male mice (data not shown, significance level set at $P < 0.05$). The MR acquisition protocol performed for this cohort of animals allowed finer anatomical mapping of structural subregions of the amygdala (see “Materials and methods”, Fig. 7a, b). As a result, signals in *Oxtr* KO and WT animals ($n = 11$) were determined among the basal, central, medial, and lateral nuclei of the amygdala in which the central nucleus of the amygdala exhibits the greatest signal intensity ($P = 0.025$, Fig. 7c). Furthermore, none of the Mn^{2+} infusion treatments was observed to produce any ostensible negative effects on animal physiology or behavior throughout the duration of manganese infusion. The additional cohort of *Oxtr* KO and WT mice infused with a 3-day Mn^{2+} treatment (total dose of 180 mg/kg, $n = 6$) showed no significant differences in daily body weights (data not shown, significance levels set at $P < 0.05$).

Discussion

The ability of MEMRI to produce images of enhanced contrast not only promotes this technique as a brain morphology imaging tool, but also as a functional imaging strategy due to manganese's selective accumulation in more active regions of the brain. Consequently, MEMRI provides us the opportunity to measure neuronal activity that occurred prior to MR acquisition. This feature allows functionally imaged animals to behave naturally in their familiar environments—an advantage over BOLD fMRI, which restricts animal movement in an unfamiliar setting (Ferris et al. 2008). However, the need to explore less physiologically noxious and behaviorally disruptive methods of manganese administration is crucial when one considers the necessity for functional studies to be conducted on healthy, normally behaving animals. The lack of comprehensive work that explores how rate and duration of continuous slow infusion of manganese affect signal enhancement is a significant issue considering that animal behavioral trials may range in duration from minutes to several days.

In this study, we demonstrated the successful application of continuous slow systemic infusion of manganese via mini-osmotic pump administration in a transgenic *Oxtr* KO species and in control, C57BL/6J adult male mice. Using this contrast agent delivery technique, we observed clear evidence of Mn^{2+} accumulation in regions throughout the entire mouse brain. It is of particular interest to note manganese's preferential accumulation in sensory and limbic areas of the brain, regions that are active during exploratory, emotional, and memory-related activities. These findings support previous work examining manganese's role as a functional marker since regions of greater neuronal activity will take up naturally more Mn^{2+} present in the surrounding interstitial fluid by way of more frequent voltage-gated calcium ion channel openings.

The lack of significant differences in signal mean measurements calculated from the 3-, 7- and 14-day treatments indicate that each of these infusion periods (with fixed cumulative

Mn²⁺ doses) are comparable in ultimate signal intensity observed in the mouse brain. Signal means for each of the examined ROI's suggest an increasing trend in relation to higher rates of MnCl₂ infusion (though not statistically significant in this study). It is likely that the greater the concentration gradient of manganese present between the extracellular space and the neuronal cytoplasm, the more facilitated entry of Mn²⁺ into active neurons occurs; subsequently, increasing signal enhancement at higher fractionated doses (Bock et al. 2008). However, this study's employment of a steady, continuous administration of Mn²⁺ via systemic osmotic infusion reduces consequently the effective concentration gradient at any point in time compared to that from an acute fractionated dose injection (given that the biological half-life of Mn²⁺ in the brain exceeds 1.5 months) (Takeda et al. 1995). As a result, measurements in mean signal intensity are expected to be similar for all Mn²⁺ treatments of equal cumulative doses regardless of infusion rate for scans collected at the completion of Mn²⁺ administration—as was the case in this study. This phenomenon regarding infusion-based MEMRI distinguishes this technique from existing fractionated dose injection methods and is therefore a key factor to consider while employing this tool to examine functional activity in response to behavioral assays of varying durations.

More detailed anatomical mapping performed on a cohort of normally behaving 3-day Mn²⁺ infused mice imaged at a higher spatial resolution found significantly greater signal measurements in the central nucleus of the amygdala compared to that of the basal, lateral, and medial nuclei of the amygdala in both *Oxtr* WT and KO animals. This finding is important to note considering the role of the central nucleus of the amygdala in relaying signals associated to routine behaviors such as feeding and reward (Ahn and Phillips 2002), and further supports manganese's mechanism of cellular entry as a function of neuronal activity.

The continuous, yet slow, release of Mn²⁺ via osmotic pump did not induce any observable toxic effects on animal physiology or behavior, in agreement with the previous work examining Mn²⁺ infusion in rats (Eschenko et al. 2010). In this current study, however, we achieved a significantly higher dose of 180 mg/kg, the greatest non-lethal manganese dose tested previously in rodents. The absence of observable deleterious health effects in any of our treatment groups, in addition to our finding that daily body weight does not change following a 3-day Mn²⁺ administration, suggests that cumulative doses of greater than 180 mg/kg may be administered in mice via mini-osmotic pump infusion without significant behavioral or physiological deficits to the animal. It is important that future studies include controls to discern subtle effects that Mn²⁺ infusion may have on behaviors.

Future studies

To assess neuronal activity in any transgenic animal model, it is critical that measurements of basal levels of Mn²⁺ accumulation in the brain be first determined and compared to that of wildtype or control strains. Once this has been established, it is possible for differences in brain functional activity to be resolved following behavioral testing. Therefore, a future goal of this preliminary work is to employ infusion-based MEMRI via mini-osmotic pump administration during behavioral manipulations of *Oxtr* KO and WT mice, and to search for differences in Mn²⁺ accumulation in regions of the brain of significant *Oxtr* density. From this present work's establishment of baseline Mn²⁺ accumulation levels in the brains of *Oxtr* KO and control C57BL/6J mice, variations in signal activity in *Oxtr* KO animals following behavioral testing will be assessed in forthcoming studies.

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Fig. 1.

MnCl₂ treatment timeline for the three infusion periods (3-, 7-, and 14-day). An initial pre-contrast scan preceded each infusion treatment, and a final post-contrast scan concluded each treatment period. *Oxtr* knockout and control C57BL/6J adult male mice were tested in each treatment group ($n = 4$ per genotype). Total cumulative doses of 180 mg/kg of MnCl₂ were administered in each group via mini-osmotic pump infusion

Fig. 2.

T1-weighted whole-brain 3-D MRI (100 μm isotropic resolution) of a control C57BL/6J adult male mouse after 3-day MnCl_2 infusion (180 mg/kg cumulative dose). Representative coronal (a), sagittal (b), and horizontal (c) slice images demonstrate Mn^{2+} accumulation as enhanced signal throughout the entire brain

Fig. 3.

Selected horizontal (**a**), sagittal (**b**), and coronal (**c**) T1-weighted slice images (100 μm isotropic resolution) from a control C57BL/6J adult male mouse brain following a 3-day MnCl_2 infusion (180 mg/kg cumulative dose). Structures of significant Mn^{2+} accumulation include the olfactory bulbs (OB), areas of the cortex (Cx) (i.e., infralimbic cortex (IL), motor cortex, visual cortex), habenula (Hb), hippocampal formation (HF) (i.e., CA1, CA2, CA3, dentate gyrus), cerebellum (Cb), inferior colliculus (IC), central amygdala (CeA), pituitary (Pt), thalamus (Th), and hypothalamus (Hy). 3-D whole-brain composite model demonstrates location from which representative slice images were selected (**d**)

Fig. 4.

ROI demarcations of the bilateral olfactory bulbs, hippocampal formation, and amygdalae on T1-weighted coronal slice images (isotropic spatial resolution = 100 μ m, field of view = $1.56 \times 1.5 \times 1.2$ cm, image matrix = $256 \times 150 \times 120$)

Fig. 5.

Normalized mean signal comparisons of olfactory bulb, hippocampal formation, and amygdala ROI's by Mn^{2+} treatment group (equal cumulative 180 mg/kg doses). *Error bars* are SEM. Mean signal measured from T1-weighted coronal slice images. Data includes *Oxtr* knockout and control C57BL/6J mice ($n = 8$ per treatment group)

Fig. 6. Sagittal (a), coronal (b), and horizontal (c) T1-weighted slice images (100 μm isotropic resolution) from control C57BL/6J adult male mouse brains prior to and following 14-, 7-, and 3-day MnCl_2 infusion treatments (180 mg/kg cumulative doses). Progressively greater signal enhancement was observed at higher rates of Mn^{2+} infusion over shorter infusion periods

Fig. 7. ROI demarcations of amygdalar nuclei on T1-weighted coronal slice images. Selected images at two depths (**a**, **b**) demonstrate the central (CeA), lateral (LA), basolateral (BLA), and medial (MeA) nuclei of the amygdala (spatial resolution = $75 \times 75 \times 150 \mu\text{m}$, field of view = $1.4 \times 1.4 \times 0.48 \text{ cm}$, image matrix = $192 \times 186 \times 32$). (**c**) Signal intensity (normalized means per unit area, arbitrary units) comparisons by amygdalar nucleus. *Error bars* are SEM. Mean signal measured from T1-weighted coronal slice images following 3-day Mn^{2+} infusion treatment (180 mg/kg cumulative dose). Data includes *Oxtr* KO and wildtype mice ($n = 12$). Asterisk indicates that signal intensity was greater in the central nucleus of the amygdala compared to that in the other nuclei ($P < 0.05$)