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Potential of N-acetylated-para-aminosalicylic Acid to Accelerate Manganese Enhancement Decline for Long-term MEMRI in Rodent Brain

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Abstract

Background—Manganese (Mn^{2+})-enhanced MRI (MEMRI) is a valuable imaging tool to study brain structure and function in normal and diseased small animals. The brain retention of Mn^{2+} is relatively long with a half-life ($t_{1/2}$) of 51 to 74 days causing a slow decline of MRI signal enhancement following Mn^{2+} administration. Such slow decline limits using repeated MEMRI to follow the central nervous system longitudinally in weeks or months. This is because residual Mn^{2+} from preceding administrations can confound the interpretation of imaging results. We investigated whether the Mn^{2+} enhancement decline could be accelerated thus enabling repeated MEMRI, and as a consequence broadens the utility of MEMRI tests.

New Methods—We investigated whether N-acetyl-para-aminosalicylic acid (AcPAS), a chelator of Mn^{2+} , could affect the decline of Mn^{2+} induced MRI enhancement in brain.

Results and Conclusion—Two-week treatment with AcPAS (200 mg/kg/dose $\times$ 3 daily) accelerated the decline of Mn^{2+} induced enhancement in MRI. In the whole brain on average the enhancement declined from 100% to 17% in AcPAS treated mice, while in PBS controls the decline is from 100% to 27%. We posit that AcPAS could enhance MEMRI utility for evaluating brain biology in small animals.

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Comparison with Existing Methods—To the best of our knowledge, no method exists to accelerate the decline of the Mn^{2+} induced MRI enhancement for repeated MEMRI tests.

Keywords

Manganese enhanced MRI (MEMRI); N-acetylated-para-aminosalicylic acid (AcPAS); Chelation; Repeated MEMRI

Introduction

Manganese enhanced MRI (MEMRI) is a powerful imaging tool to measure rodent neural structure, function and linked pathways (1–3). It is facilitated by manganese ion (Mn^{2+}) entry into neurons that occurs through calcium channels. Here, ions accumulate in neurons and are transported along axons. Such ion trafficking reflects neuronal function and networks (1–5). Mn^{2+} is an excellent T_1 shortening paramagnetic contrast agent inducing signal enhancement on T_1 -weighted (T_1 -w) MRI. The retention of Mn^{2+} in brain is prolonged with a half-life ($t_{1/2}$) of 51 to 74 days (5). Such ion retention allows studies of brain function performed on non-restrained awake rodents (6–10). In this context, Mn^{2+} administration, sensory stimulation and/or behavioral tests are performed outside the MRI scanner and prior to imaging tests. Studies have shown that brain activities that took place days before imaging tests could be detected using appropriate Mn^{2+} administration schemes such as implanted osmotic pump infusion (9, 10). The long Mn^{2+} brain retention time also enables the monitoring of Mn^{2+} axonal transportation for studies evaluating neural pathways (11–13). MEMRI is also proven a valuable tool in neurodegenerative disorder studies using rodents (14–25). However and despite such advantages, its application is limited in the longitudinal follow-up of neurodegenerative disorders due to the prolonged washout time of Mn^{2+} . During the time span of the studies that can be as long as weeks and even months, repeated Mn^{2+} administration is usually necessary to keep brain Mn^{2+} concentration consistent among imaging sessions. This requires carefully designed dosing if imaging needs to be performed before the residual Mn^{2+} from *preceding* administrations is eliminated. Infusion using commercially available osmotic pumps may keep brain Mn^{2+} concentration consistent for up to six weeks (Alzet, Cupertino, CA), which is usually not sufficient in time to evaluate the progression of neurodegenerative disorders in rodents. Moreover, repeated or continuous Mn^{2+} administration can cause secondary toxicities (26). One solution is to accelerate Mn^{2+} brain elimination after each MEMRI tests and as such limit the effect of residual Mn^{2+} on the MEMRI evaluation. Accelerated Mn^{2+} washouts may also serve to minimize Mn^{2+} toxicity.

With this in mind, we tested whether N-acetylated-para-aminosalicylic acid (AcPAS) could accelerate Mn^{2+} elimination from brain. AcPAS, an N-acetylated metabolite of para-aminosalicylic acid (PAS), was previously used to treat human manganism, a disorder which parallels several of the clinical features of Parkinson's disease (27). Treatment of Mn^{2+} intoxication is linked to PAS chelation (28, 29). Chelation is the binding of organic compounds and metal ions. The brain distribution, metabolism, and time-concentration relationships of PAS and its major metabolite, AcPAS, were previously investigated (30, 31). The results demonstrated that AcPAS chelates Mn^{2+} . AcPAS has higher brain

concentration and possesses a longer $t_{1/2}$ than PAS. Herein we demonstrate that AcPAS can be employed to improve the MEMRI utility by permitting serial brain measurements in health and disease.

Materials and Methods

Study Design

C57BL/6 mice were used in this study. Mice were housed in the University of Nebraska Medical Center (UNMC) laboratory animal facility according to the American Animal Association and Laboratory Animal Care guidance. All procedures were approved by the Institutional Animal Care and Use Committee at UNMC. The kinetics of AcPAS in brain tissue and plasma was first studied using high-performance liquid chromatography (HPLC) using one group of mice. Another group of mice was first administered MnCl_2 via the intraperitoneal (i.p.) route, followed with PBS ($n = 3$), low dose ($n = 3$, 100 mg/kg), medium dose ($n = 3$, 150 mg/kg) and high dose AcPAS ($n = 3$, 200 mg/kg) three times daily for two weeks. The doses and administration scheme were designed based on the previous PK studies of AcPAS (30–32). MRI was performed one day after the MnCl_2 administration followed by AcPAS/PBS treatment. Two more MRI scans were performed at one and weeks of AcPAS/PBS treatment. After the last MRI, the mice were immediately euthanized for inductively coupled plasma mass spectrometry (ICP/MS) analysis of brain Mn^{2+} concentrations. The timeline of the study design is shown in Fig. 1. Three animals were randomly selected from the above 12 AcPAS/PBS-treated mice and were scanned before any drug administration for baseline measurements of MRI and ICP/MS.

AcPAS Synthesis

AcPAS was synthesized by a modified procedure (33). Briefly, p-aminosalicylic acid (0.33 mol), was dissolved in 100 ml of 2 M hydrochloric acid and stirred with sodium acetate (0.33 mol) in water at 0° C. The reaction mixture was stirred overnight with 50 ml of acetic anhydride at room temperature. The brown precipitate obtained was filtered, washed, dried and dissolved in 0.1M sodium hydroxide then stirred overnight. The resulting solution was adjusted to pH 2 with HCl. The product was extracted with ethyl acetate (3×75 ml) and the extracts were dried over anhydrous sodium sulphate. The solid residue was washed with hexane to produce 52 % yield of pure AcPAS. The identity of AcPAS was confirmed by NMR with > 99 % purity.

High performance liquid chromatography (HPLC)

AcPAS (200 mg/kg) was administered to mice ($n = 9$) by i.p. injection. Plasma was collected at 0.5, 1, 2, 6 and 12 hours. Mouse peripheral blood samples (100 μl) were collected from submandibular vein (cheek bleed) by using sterile lancets (MEDIpoin, Inc., Mineola, NY) in EDTA coated tubes. Plasma was separated by centrifugation of blood samples at 1,800 rpm for 8 min at 4°C within 1 h of sample collection and stored at -80°C until analysis. Brain tissues (cortex, thalamus, olfactory bulb) of same group of mice administered with the same dose of AcPAS were collected at 6, 8 and 12 ($n = 3$ for each time point) hours after administration. AcPAS from plasma and tissues were extracted using acetonitrile. AcPAS was quantified by HPLC according to the method of Sugamori et al.

(34). Briefly, extracted plasma or brain samples were injected in duplicate (20 μ l) onto a Shimadzu HPLC (Shimadzu Scientific Instruments, Columbia, MD) containing a Synergi 4 μ Hydro-RP column (4.6 $\times$ 150 mm) (Phenomenex, Torrance, CA) and extruded using an isocratic mobile phase of 7% acetonitrile/1% acetic acid/0.1% triethylamine at a flow rate of 1.6 ml/min. AcPAS were detected at a wavelength of 270 nm and quantitated by peak comparisons to a standard curve (0.05–50 μ g/ml).

MnCl₂ and AcPAS treatment

MnCl₂·4H₂O (Sigma-Aldrich, St Louis, MO) was added to 0.9% w/v NaCl₂ (Hospira, Lake forest, IL) to make 50 mM MnCl₂ solution. MnCl₂ solution was delivered i.p. at a dose of 60 mg/kg consecutively four times at 24 hour intervals. The fractional administration scheme was designed to minimize Mn²⁺ toxicity (35). After injection, the mice were observed daily to monitor potential Mn²⁺ toxicities. AcPAS injection started one day after the MnCl₂ administration. AcPAS (1 mg) was mixed with 1 μ l DMSO in a glass container. More (1–2 μ l) DMSO was added until the compound was dissolved. Physiological saline (0.9% NaCl) was added to dilute the solution to 20 mg/ml AcPAS. The solution became a suspension with addition of saline. It was then stored at 4 °C. Before each injection, the suspension was thoroughly mixed by shaking. AcPAS was injected i.p. 3 times per day at eight-hour intervals for two weeks. Before each drug injection, the animal was observed for any abnormal changes in behavior, hair coat, facial expression, ambulation and body weight.

MRI

MRI was performed on a Bruker Bioscan 7 Tesla/21 cm MRI (Bruker, Billerica, MA) operating Paravision 5.1 with a 82 mm quadrature volume resonator and a 4-channel phased array receive coil. Mice were anesthetized by inhalation of isoflurane in 100% oxygen and maintained 40–80 breaths/minute. Mice were scanned using T₁ mapping (fast spin echo with variable TR from 0.4 s to 10 s, TE = 7.0 ms, 12 coronal slices, slice thickness = 0.5 mm, in-plane resolution = 0.156 $\times$ 0.156 mm², FOV = 20 $\times$ 20 mm²) and three-dimensional T₁W MRI (gradient recalled echo, TR/TE = 20/4.5 ms, flip angle = 20°, FOV = 19.2 (Left - Right) $\times$ 19.2 (Superior - Inferior) $\times$ 30.0 (Anterior - Posterior) mm³, spatial resolution = 0.1 $\times$ 0.1 $\times$ 0.1 mm³).

To reduce the influence of the inhomogeneous signal reception on the T₁W images by the phased array surface coil, N3 field inhomogeneity correction (36) was first performed on each image using MIPAV (CIT, NIH). The brain volumes in the T₁W images were extracted using an in-house Matlab program(37) based on the level sets method. The brain images were then registered to the MRI-based mouse brain atlas downloaded from the Laboratory of Neuro Imaging (LONI) at the University of Southern California using affine transformation first, and then nonlinear transformation (DiffeoMap, John Hopkins University, Baltimore, MD).

To use signal changes in high resolution T₁W MRI to represent Mn²⁺ accumulation, signal differences need to be scaled to absolute changes in T₁ values. This is achieved by calibrating the baseline and post Mn²⁺ injection T₁W images using T₁ values. The T₁ maps were first generated using an in-house Interactive Data Language (IDL) version 8.2 (Exelis

Visual Information Solutions, Boulder, Colorado) program from the data acquired by T₁ mapping sequence. Regions of interest (ROI) were then placed on relatively uniform tissue regions including frontal cortex and caudate on T₁ maps and T₁W images. *The baseline and post Mn²⁺ injection longitudinal relaxivity and T₁W signal intensity in the ROIs were measured. We assumed that the effect of the system variation is a constant C (calibration factor) and set system parameter of the post- manganese session as 1, then the measured baseline signal (designated as S_{bl}^C) is $S_{bl}^C = CS_{bl}$, where S_{bl} is the true (calibrated) baseline signal. The calibration factor was calculated as $C = (S_{bl}^C/S_{Mn}) \times (R_{1Mn}/R_{1bl})$, where R_{1bl} and R_{1Mn} are baseline and post Mn²⁺ injection longitudinal relaxivities, respectively; S_{Mn} is post Mn²⁺ T₁W signal intensity. The calibration factor was then applied to the baseline T₁W image to calculate the true baseline image: $S_{bl} = S_{bl}^C / C$.*

The Mn²⁺ induced T₁W signal enhancement was calculated by: $(S_{Mn} - S_{bl}) / S_{bl}$. This equation was derived by ignoring the T₂* effect in the MRI signal generated using a spoiled gradient recalled echo, and replacing the exponential of $(-TR/T_1)$ with the first two terms of its Taylor expansion. These operations were appropriate as a previous study has shown that Mn²⁺ induced T₂ change is small compared to T₁ reduction (38), and $(-TR/T_1)$ is small (TR = 20 ms and T₁ > 800 ms in most brain regions). Using the LONI brain atlas, enhancement on brain regions/sub-regions was calculated. A region-by-region comparison was performed between AcPAS treated groups and PBS controls using Student's t-test at each time point.

ICP/MS Analyses

Known weights of thawed brain regions (cortex, thalamus, olfactory bulb), collected from the animals were decomposed by wet-ashing in vials with six volumes of concentrated nitric acid followed by microwave (MARS, CEM Corp., Matthews, NC) heating at 200 °C. Total manganese concentrations were determined by ICP-MS (NexION 300Q, PerkinElmer, MA, USA).

Statistical Analysis

Student's t-tests were used for all statistical analyses. The significance level was 0.05.

Results

AcPAS plasma and brain levels

AcPAS plasma and brain concentrations are shown in Figure 2. AcPAS was reduced in concentration at 2 hours from > 400 to < 23 ng/ml in plasma. The reduction reached eight ng/ml at 12 hours. AcPAS increased in the cortex, thalamus and olfactory bulbs with time after six hours. The highest AcPAS tissue concentration was in olfactory bulbs with less drug in the thalamus and cortex (Fig. 2).

MRI

Mn²⁺ induced enhancement on T₁W MRI was seen after 24 hours of MnCl₂ injection (Fig 3A–a). Mn²⁺ induced signal enhancement on T₁W MR images was seen in the hippocampus, cerebellum and olfactory bulbs (Fig 3A–a)(3, 4). The MR images one week after MnCl₂ injection are illustrated in Fig 3A–b with the top panel the PBS and bottom high

dose AcPAS treated mice. The signal intensity was decreased at 1 week in both groups. Compared to PBS controls, decreased signal intensities were seen in AcPAS treated mice. This was most notable in the hippocampus and cortex. After 2 weeks of treatment, AcPAS mice (Fig 3A–c, top panel) showed greater decreased signal intensity than PBS controls (Fig 3A–c, bottom panel) throughout the multiple brain regions.

Mn²⁺ enhancement

The enhancement maps are shown in Fig 3B, and quantified region-specific enhancement results are listed in Table 1. After 24 hours, Mn²⁺ induced enhancement is throughout the whole brain (96% enhancement) with the relatively high enhancement on olfactory system (Olf, 97%), lateral olfactory tract (lo, 136%), optic chiasm (ox, 98%), hippocampus (HIP, 97%), hypothalamus (Hy, 98%), interpeduncular nucleus (IP, 97%), and cerebellum (Cb, 96%) regions (Fig 3B–a, 1st column in Table 1). The brain region abbreviations follow the conventions in the Paxinos and Franklin mouse brain atlas (39). Natural enhancement decline was clearly seen in PBS controls after one week from 85–136% to 30–62% (Fig 3B–b top panel, 2nd column in Table 1). Mice treated with low (100 mg/kg) and medium (150 mg/kg) doses did not show significant difference compared to PBS controls (data not shown). The enhancement in high dose (200 mg/kg) AcPAS treated mice was lower than in PBS animals (Fig 3B–b). The enhancement difference was significant ($p < 0.05$) on substantia nigra (SN), corpus callosum (cc), thalamus (Th), hippocampus (HIP) and olfactory bulb (Olf) regions (3rd column in Table 1).

The enhancement decreased significantly after two weeks in PBS controls to 22–59% (Fig 3B–c top panel, 4th column in Table 1). After two-weeks, medium dose (150 mg/kg) AcPAS treated mice showed significantly decreased enhancement compared to PBS controls on fornix (f) and optic chiasm (ox) (5th column in Table 1). In high dose (200 mg/kg) AcPAS treated mice, a number of brain regions showed significantly decreased enhancement including the caudate putamen (CPu), basal ganglia (BG) and internal capsule (ic) (6th column in Table 1). The whole brain enhancement in high dose (200 mg/kg) AcPAS treated mice was also significantly less than in PBS controls. The brain regions with significantly reduced enhancement in high dose (200 mg/kg) AcPAS treated mice are shown in Fig 4.

Manganese concentrations measured by ICP/MS in mouse brains at 24 hours after MnCl₂ administration were 183±25.8 parts per billion (ppb) in cortex, 280±29.2 ppb in thalamus, 203±14.8 ppb in hippocampus, and 507±164 ppb in olfactory bulbs. The concentrations were presented as mean±SEM. The concentrations decreased in PBS treated controls after 2 weeks to 61.6±1.45, 71.7±2.96, 57.6±7.61 and 170±21.4 ppb in cortex, thalamus, hippocampus and olfactory bulbs, respectively. The concentrations in high dose AcPAS treated mice at two weeks were 74.3±9.29, 85.3±22.2, 72.8±26.6, and 119±19.4 ppb in cortex, thalamus, hippocampus and olfactory bulbs, respectively. No significant difference was found between saline controls and AcPAS treated mice ($p > 0.1$, *data not shown*).

Discussion

This study investigated the abilities of AcPAS treatment to affect repeated MEMRI measurements of brain structure and function. We demonstrate that, even though AcPAS did

not lead to accelerated Mn^{2+} brain elimination, it suppressed significantly Mn^{2+} -induced MRI enhancement after about two weeks treatment when using the high dose ($200\text{mg/kg} \times 3$ daily). Based on these findings, AcPAS has the potential to enable repeated MEMRI measurements and minimize Mn^{2+} toxicity.

AcPAS can be rapidly eliminated from blood within an hour. As soon as six hours after the injection, AcPAS has entered the brain and its concentration increased over the next six hours. This finding is in agreement with a previous PK study of PAS and AcPAS (32). These findings suggested that, to achieve sufficient brain concentrations, AcPAS must be administrated by multiple injections over a day or by continuous infusion. Therefore the dosing and administration scheme were designed based on these findings and previous PK and PD studies of AcPAS (32). The accelerated Mn^{2+} washout induced by AcPAS is different among brain regions. HIP, TH, CPu are among the regions of fastest washout. This is in the agreement of a previous study showing high AcPAS concentration in these regions (30).

The ICP/MS data suggested that AcPAS failed to speed the elimination of Mn^{2+} . However, MRI results showed faster enhancement decline in AcPAS treated mice compared to PBS controls at high dose. The discordant results can be explained as follows. The chelation of paramagnetic Mn^{2+} by AcPAS limits its interaction with water molecules, and thus causes the decrease in Mn^{2+} induced signal enhancement. On the other hand, the high tissue affinity and long $t_{1/2}$ of AcPAS limit the elimination of chelated Mn^{2+} from brain.

The study showed that an average 17% enhancement remains in brain after 2-week high dose AcPAS treatment. Further experiments are necessary to determine the time to complete elimination of enhancement. Nevertheless, AcPAS provides an option for serial studies with a shorter time interval compared to the inherent Mn^{2+} washout of 17–26 weeks (5). We did not observe any side-effects of AcPAS in the animals. It is quite possible that higher dose ($> 200 \text{ mg/kg}$) AcPAS treatments would eliminate the enhancement more quickly than the highest dose used in this study.

A more comprehensive study is being planned, in which a second MEMRI experiment will be performed after AcPAS treatment to ensure the enhancement efficiency still holds, and Mn^{2+} uptake and retention by neurons are not affected after the AcPAS treatment. The study will prove the feasibility of using AcPAS for repeated MEMRI. We do not expect AcPAS treatment causes brain water relaxivity changes, because AcPAS is an organic compound cleared from brain with a half-life of 100–200 mins (30), and is diamagnetic. To prove the hypothesis, one more group of mice will be added in the planned study that will receive AcPAS treatment and MRI scans but not MnCl_2 injection. This group is to detect any effect on brain water relaxivity caused by AcPAS treatment.

PAS [4-amino-2-hydroxybenzoic acid (Paser); CAS number, 65–49–6] has been well known as an antituberculosis drug since the 1950s (40). Its side effects on humans have been investigated (40). The toxicity of its tissue metabolite - AcPAS needs to be further studied for use in MEMRI. In this study, no abnormal changes in behavior, hair coat, facial expression, ambulation and body weight were found in the animals. Even though AcPAS

does not accelerate the removal of Mn^{2+} from brain, its chelation of Mn^{2+} is believed to minimize Mn^{2+} toxicity. If AcPAS is to be used in neurological disease studies using rodent models, its compatibility with the studies needs to be considered to assure its administration will not advertently affecting key physiological/disease-related parameters. In conclusion, we showed that, while AcPAS does not eliminate Mn^{2+} from brain, it accelerates Mn^{2+} induced enhancement decline in MRI. The results suggested the potential to use AcPAS for serial MEMRI measurements of voltage-gated calcium channel activity and morphology in rodent brains. In future studies, we will follow the animals longer to study the long-term effects of AcPAS on Mn^{2+} elimination and parallel MEMRI test results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

f	fornix
ox	optic chiasm
Cb	cerebellum
SN	substantia nigra
IP	interpeduncular nucleus
cc	corpus callosum
Hy	hypothalamus
Th	thalamus
CPu	caudate putamen
BG	basal ganglia
HIP	hippocampal region
lo	lateral olfactory tract
Olf	olfactory system
Cx	cerebral cortex
ic	internal capsule
fr	fasciculus retroflexus
Br	whole brain

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Highlights

- We tested AcPAS for its abilities to accelerate time-dependent decline in Mn^{2+} enhancement for MRI tests conducted in rodent brains
- A 2-week AcPAS treatment accelerated the declines in MRI Mn^{2+} enhancements
- AcPAS treatment enables repeated MEMRI tests during dynamic long-term studies of brain morphology and function.
- AcPAS treatment does not accelerate Mn^{2+} brain excretion.

Figure 1.

Study design. Mice were first administrated with MnCl_2 , followed with PBS ($n = 3$), low dose ($n = 3$, 100 mg/kg), medium dose ($n = 3$, 150 mg/kg) or high dose AcPAS ($n = 3$, 200 mg/kg) for two weeks. MRI was performed on the mice at one and two weeks after MnCl_2 administration. After the second MRI, the mice were immediately euthanized for ICP/MS analysis of brain Mn^{2+} concentrations.

Figure 2. AcPAS concentrations measured by HPLC in plasma (blue line) and in the brain regions (red lines). The concentrations in plasma were measured in ng/ml (left vertical axis), and in brain were measured in ng/mg (right vertical axis). The error bar at each time point shows the mean standard error of the measurement.

Figure 3.

(A) MRI data. (a) Averaged MRI of mice ($n = 9$) at 24 hours after MnCl_2 administration. (b) Averaged MRI of PBS controls ($n = 3$, top panel) and high dose AcPAS treated mice ($n = 3$, bottom panel) at one week after MnCl_2 administration. (c) Averaged MRI of PBS controls ($n = 3$, top panel) and high dose AcPAS treated mice ($n = 3$, bottom panel) at two weeks after MnCl_2 administration. (B) Manganese enhancement maps corresponding to Figure 3A.

Figure 4.

Brain regions with significantly less enhancement in mice after one (A) and two (B) weeks high dose ACPAS treatment compared to in PBS controls. The regions are shown in coronal slices, whose locations are indicated using red lines on the sagittal slices in the leftmost column.

Table 1

Brain regions with significantly decreased enhancement in mice after 1 and 2 weeks high dose AcPAS treatment compared to in PBS controls.

	0 week			1 week			2 weeks		
	All (%)	PBS (%)	High Dose (%)	PBS (%)	High Dose (%)	PBS (%)	Mid Dose (%)	High Dose (%)	High Dose (%)
f	89±13	35±7	27±7	29±2	21±0**	21±0**	21±3	18±6**	18±6**
Ox	98±14	37±11	37±7	35±6	23±3**	23±3**	21±3	22±5**	22±5**
Cb	96±13	39±7	32±7	28±6	21±3	21±3	21±3	19±4*	19±4*
SN	91±12	40±4	30±7**	22±8	16±4	16±4	24±2	12±5*	12±5*
IP	97±14	50±3	37±11*	31±9	24±2	24±2	24±2	18±7*	18±7*
cc	87±12	37±4	27±6**	25±6	17±2*	17±2*	17±2*	15±4**	15±4**
Hy	98±13	41±5	37±5	33±7	23±2*	23±2*	23±2*	17±5**	17±5**
Th	89±13	38±4	28±5**	25±7	20±1	20±1	20±1	16±5*	16±5*
CPu	90±12	40±4	30±8*	29±7	20±1*	20±1*	20±1*	17±4**	17±4**
BG	95±13	42±4	33±8*	31±8	21±1	21±1	21±1	17±6**	17±6**
HIP	97±13	42±4	33±6**	29±8	20±0	20±0	20±0	17±5**	17±5**
lo	136±24	62±20	60±11	59±12	45±2	45±2	45±2	39±9**	39±9**
Olf	97±13	45±8	31±8**	28±5	22±1	22±1	22±1	20±6*	20±6*
Cx	95±14	40±8	32±6*	29±8	23±0	23±0	23±0	18±4*	18±4*
ic	85±16	30±4	24±7	22±6	16±6	16±6	16±6	13±4**	13±4**
fr	95±17	38±6	32±4*	27±10	23±5	23±5	23±5	17±6	17±6
Br	96±16	36±9	29±7	27±6	22±2	22±2	22±2	17±5**	17±5**

Enhancement data are presented as percentage and in Mean ± Standard deviation.

* p < 0.1;

** p < 0.05