# Investigation of hypothalamic neuronal and metabolic mechanisms of anorexia with Manganese-enhanced MRI and Proton MR Spectroscopy

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**Introduction:** In order to understand the organization, interaction and function of neuronal pathways controlling feeding in normal and anorexic states, the identification of important neurons and their connections is essential. Dehydration-induced anorexia (DIA) is a validated experimental model (1) allowing investigation of the neuronal mechanisms of anorexia in rodents. Here, Manganese-Enhanced MRI (MEMRI) and proton MR spectroscopy were used to investigate hypothalamic neuronal and metabolic differences between dehydrated and overnight fasted (OFS) rats.

**Materials and Methods:** 36 female Wistar rats (225-250g body weight (b.w) on Day 0 (D0)) were divided into 3 groups: the Control group (CTL, n=12), the overnight food suppressed group (OFS, n=8) and the dehydration induced anorexia group (DIA, n=16). On day 0 (D0), animals from the CTL group and the OFS group received water and food *ad libitum;* the dehydrated group (DIA) received 2.5% NaCl (SIGMA, Zofingen, Switzerland) as drinking solution (1). On D3, OFS rats had their chow removed until the MR scan took place, 24 hours later. On D3, rats from each group were anesthetised with 2% isoflurane in a mixture of $O_2$ and $N_2O$ via a facemask. Each rat received an i.v infusion of 100 mg/kg at 100mM $MnCl_2$ at a rate of 0.5ml-1ml/h via the tail vein to avoid cardiac arrest. For proton magnetic resonance studies, 5 female Wistar rats per group were used. These rats did not undergo $MnCl_2$ infusions and were scanned on D4 after the start of anorexic protocol (D0) for the DIA group of rats.During the MR experiments, rats were anesthetized with 2% isoflurane in a mixture of $O_2$ and $N_2O$. The rats were positioned in a dedicated holder with head fixation. Their body temperature was maintained at 37.5±0.5ºC using a temperature-controlled water circulation and a rectal probe. Their respiration rate was monitored throughout the experiment. MRI was performed on D4 24 hours after $MnCl_2$ infusion. ***MRI data acquisition:*** All the experiments were performed on a 14.1T/26cm horizontal bore magnet (Magnex Scientific, Oxford, UK). The magnet was equipped with 12-cm inner-diameter actively shielded gradient sets (Magnex Scientific, Oxford, UK) allowing a maximum gradient of 400mT/m in 120µs. A home-built quadrature T/R 17-mm surface coil was used. Field homogeneities were adjusted using an EPI version of FASTMAP (2). After the preliminary adjustments (tuning, shimming, acquisition of scout images for accurate positioning) 3D gradient echo T1-weighted images (TR/TE=20/5ms, Flip angle=70º, FOV=25x25x25mm$^3$, matrix size=256x256x128, coronal slices, BW=25 KHz, 5 averages) were acquired. ***Image Analysis:*** MR images were processed and analyzed using Image processing software (Image J 1.3.1_13. NIH, USA) and Matlab (Matlab 7.4). The MathWorks, USA). All the images were bias field corrected (3). Signal to noise ratio and t-score pixel-by pixel maps were constructed as described in (4). ***Proton MRS in the hypothalamus:*** Localized Proton spectroscopy was done using SPECIAL(5) with outer volume suppression and VAPOR water suppression (6). A voxel of interest (VOI) of 24 µl was localized in the Paraventricular Nuclei (PVN) region for all the rats using the previously acquired 3D-GRE images. All 1$^{st}$ and 2$^{nd}$ order shims were re-adjusted using FASTMAP (2) in the VOI which resulted in water linewidths of 23 ± 5 Hz. To obtain adequate signal to noise ratio (SNR), 640 scans were acquired. For absolute quantification, water signal was acquired using identical parameters, except without water suppression and averaging 8 scans. ***Quantification and statistics:*** The *in -vivo* $^1$H MR spectra were processed using LCmodel (7). In this study, metabolites concentrations were quantified using databases of simulated spectra of metabolites (7). All data are presented as mean ± standard error of the mean (S.E.M). Unpaired Student t-tests were applied to compare data acquired in the three groups of rats.

**Results:** 24-hours post-$MnCl_2$ infusion, increased signal enhancement was seen in 3D-$T_1$-weighted axial images (Fig1.A) in hypothalamic areas of DIA and OFS rats compared to CTL rats. A zoom in an area encompassing the hypothalamus (Dashed white box in Fig1.A) revealed no specific $MnCl_2$ enhancement in CTL whereas the lateral hypothalamus (LH), the paraventricular nuclei (PVN) and the arcuate nucleus (Arc) surrounding the 3$^{rd}$ ventricle (3V) showed increased signals in DIA and OFS rats (Fig1.B). These enhancements were further confirmed by the pixel by pixel SNR maps clearly demonstrating high SNR values in the hypothalamus of DIA (SNR~50-60) and of OFS (SNR~45-55) compared to CTL (SNR~30-42). Moreover, increased SNR was observed around the 3$^{rd}$ ventricle in DIA versus OFS rats demonstrating increased neuronal activation in the PVN and LH in dehydrated rats (Fig1.C). T-score maps (Fig. 2) were used to spot differences between DIA and OFS conditions in the hypothalamus. Low t-scores were detected (T-score =10-20 p>0.05) in OFS versus CTL whereas the whole hypothalamic region surrounding the 3$^{rd}$ ventricle showed significant differences with CTL (Tscore >30; p<0.05). T-scores were particularly high in the PVN and in the LH in the sub-fornical area (Tscore=40-60; p<0.015). The difference between CTL and OFS is essentially found in the PVN area (T-score=25-40; p<0.05). In DIA and OFS rats, GABA, myo-Ins and Tau levels were significantly higher than in CTL (Fig3). OFS also demonstrated significantly higher levels for total creatine (tCr, p<0.05). Lactate was significantly higher for DIA rats only.

**Discussion:** Using biochemical and histological methods, DIA and OFS rats have shown identical endocrine and neuropeptidergic profiles of negative energy balance (1). Despite free access to food, DIA rats spontaneously limit their food intake. Our MEMRI study shows increased neuronal activation of the hypothalamus in DIA and OFS but reveals that differences between DIA and OFS rats are mainly situated at the level of the PVN. However, similar neurochemical profiles were measured using $^1$H –MRS. GABA is known to suppress feeding in rats and impact on body weight loss (8). In addition, the majority of inputs to neurons of the PVN contain the inhibitory neurotransmitter GABA (9). These projections from LH convey inhibitory signals in response to dehydration and food deficiency and limit hypothalamic activity by an increase in GABA release at the level of the PVN. The similarity of OFS and DIA metabolic conditions may be explained by regulation of the hypothalamus via different hypothalamic pathways and/or by stimulation of different types of neurons present in the PVN that release identical amounts of metabolites.

**Acknowledgements:** Supported by the centre d'Imagerie Médicale (CIBM) of UNIL, EPFL, HUG, CHUV and Leenards and Jeantet foundations.

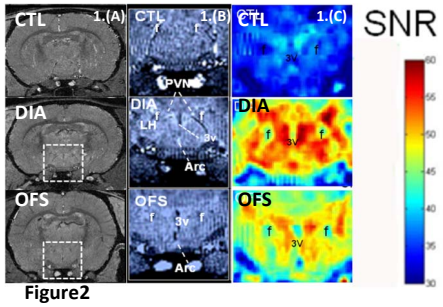
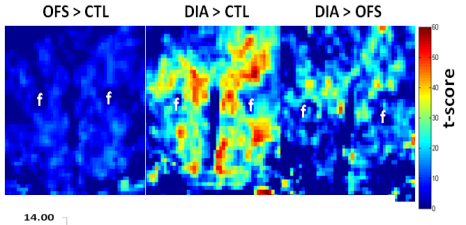



Figure2



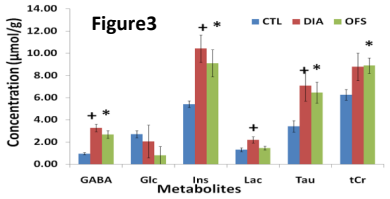




**Figure 1: (a) 3D T1-weighted images in CTL, DIA and OFS, (b) Zoom in hypothalamus represented by dashed square boxes in 1.(a) and (c) SNR maps in CTL, DIA and OFS rats. f: fornix. PVN: Paraventricular Nuclei. 3V: Third ventricle. LH: Lateral Hypothalamus. Arc: Arcuate nucleus. Figure 2: T-score maps in CTL, DIA and OFS, Figure3: Neurochemical profile comparisons. + represents statistical significance between CTL and DIA. * represents statistical significance between CTL and OFS.**

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