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Magnetic resonance spectroscopy reveals *N*-acetylaspartate reduction in hippocampus and cingulate cortex after fear conditioning

Iris Y. Zhou [a,b], Abby Y. Ding [a,b], Qi Li [c], Grainne M. McAlonan [c,d], Ed X. Wu [a,b,e,*]

[a] Laboratory of Biomedical Imaging and Signal Processing, The University of Hong Kong, Hong Kong Special Administrative Region, China
[b] Department of Electrical and Electronic Engineering, The University of Hong Kong, Hong Kong Special Administrative Region, China
[c] Department of Psychiatry, The University of Hong Kong, Hong Kong Special Administrative Region, China
[d] Department of Forensic and Neurodevelopmental Sciences, Institute of Psychiatry, King's College London, London, UK
[e] Department of Anatomy, The University of Hong Kong, Hong Kong Special Administrative Region, China

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ABSTRACT

The fear conditioning in rodents provides a valuable translational tool to investigate the neural basis of learning and memory and potentially the neurobiology of post-traumatic stress disorder (PTSD). Neurobiological changes induced by fear conditioning have largely been examined *ex vivo* while progressive 'real-time' changes *in vivo* remain under-explored. Single voxel proton magnetic resonance spectroscopy (^{1}H MRS) of the hippocampus, cingulate cortex and thalamus of adult male C57BL/6*N* mice ($N=12$) was performed at 1 day before, 1 day and 1 week after, fear conditioning training using a 7T scanner. *N*-acetylaspartate (NAA), a marker for neuronal integrity and viability, significantly decreased in the hippocampus at 1 day and 1 week post-conditioning. Significant NAA reduction was also observed in the cingulate cortex at 1 day post-conditioning. These findings of hippocampal NAA decrease indicate reduced neuronal dysfunction and/or neuronal integrity, contributing to the trauma-related PTSD-like symptoms. The neurochemical changes characterized by ^{1}H MRS can shed light on the biochemical mechanisms of learning and memory. Moreover, such information can potentially facilitate prompt intervention for patients with psychiatric disorders.

1. Introduction

Post-traumatic stress disorder (PTSD) is a highly prevalent and severe anxiety disorder triggered when a vulnerable individual experiences a highly traumatic event. In the past decade, a number of neuroimaging techniques have been employed to investigate the underlying functional and structural brain abnormalities in PTSD patients (Rauch and Shin, 1997; Hull, 2002; Hughes and Shin, 2011). In general, the findings include hyper-responsivity of amygdala (Rauch et al., 2003), hypofunction of anterior cingulate (Shin et al., 2001), and hippocampal dysfunction and volume reduction (Bremner et al., 2003; Shin et al., 2004) in patients with PTSD compared to healthy controls.

Proton magnetic resonance spectroscopy (^{1}H MRS) is a non-invasive method used to assess metabolic changes in living brain and provides biochemical clue to underlying neural pathology, even when morphological changes are not apparent. Previously, ^{1}H MRS has been used to evaluate the neurochemical changes in hippocampus and anterior cingulate cortex in PTSD patients (Mahmutyazicioglu et al., 2005; Karl and Werner, 2010). A reduction of the neuronal marker *N*-acetylaspartate (NAA) in terms of a lower NAA/creatine ratio or NAA concentration in hippocampus has been reported in patients with PTSD both with and without hippocampal volume changes compared to healthy control subjects (Schuff et al., 1997, 2001; Villarreal et al., 2002). Decreased NAA level has also been reported in the anterior cingulate cortex of subjects with PTSD. However, these changes were mainly observed in the chronic stage of disorder and compared to control groups. The pathological mechanisms of PTSD are still poorly understood. In particular, the acute changes occurring following trauma exposure and development of PTSD cannot easily be captured due to its complexity and diversity in humans (Liberzon and Martis, 2006). Therefore, animal models are indispensable in understanding the basic mechanisms of this disorder (Cohen and Richter-Levin, 2009).

The fear conditioning paradigm involves the learned association of an initially neutral conditioned stimulus (CS), such as a tone or light, with an aversive unconditioned stimulus (US), usually footshock. After a few such parings, the CS alone comes to elicit physiological and behavioral fear reactions. Because fear

* Corresponding author at: Department of Electrical and Electronic Engineering, Anatomy and Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China. Tel.: +852 2859 7096; fax: +852 2559 8738.
E-mail address: ewu@eee.hku.hk (E.X. Wu).

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conditioning has a rapid and long-lasting behavioral effect, it has been considered as a simple yet valuable model to investigate the neurobiological mechanisms of learning and memory and to understand the pathological mechanisms of fear-related disorders, such as PTSD (Lavond et al., 1993; LeDoux, 2000). Behavioral studies indicate that lesions of the amygdala or hippocampus interfere with the acquisition of conditioned fear (Phillips and LeDoux, 1992; Tanev, 2003). Consistent with this, functional neuroimaging studies have reported altered activation in amygdala and inter-connected regions such as hippocampus, anterior cingulate cortex and insular cortex are associated with fear conditioning (Rauch et al., 2006; Shin and Liberzon, 2010). These brain regions are considered to be key components in a neurocircuitry of fear conditioning (Kim and Jung, 2006). However, to date, acute *in vivo* neurochemical changes in these structures precipitated by fear conditioning have not been examined.

In this study, *in vivo* ^{1}H MRS was employed to investigate the metabolic changes in hippocampus, cingulate cortex and thalamus of mouse brain before and after fear conditioning. This study aimed to characterize the longitudinal neurochemical changes underlying fear conditioning by ^{1}H MRS and to contribute towards a clear understanding of the neurobiological mechanisms of fear learning and memory.

2. Materials and methods

2.1. Animals

All experiments were approved by the Institutional Animal Care and Use Committee. Adult male C57BL/6*N* mice ($N=12$) weighing 23–28 g were used in this study. Animals were housed in groups of four under a 12:12 h light/dark cycle and had *ad libitum* access to food and water. ^{1}H MRS measurements were performed 1 day prior to fear conditioning training, 1 day and 1 week after the training. During the imaging experiments, each mouse was anesthetized with isoflurane (with 3% induction and 1.0%–1.5% maintenance) and kept warm with circulating water at 37° C while under respiratory monitoring.

2.2. Behavioral procedure

The experimental setup for fear conditioning is custom-made according to the previous description (Barnes and Good, 2005). Briefly, the fear conditioning apparatus comprised a conditioning chamber ($25\times25\times25$ cm^3) with a grid floor made of 4 mm diameter stainless steel rods spaced at 8.9 mm apart. The chamber was entirely encased within a sound attenuating box and masking noise was provided by an extractor fan. An infrared digital camera was mounted 50 cm directly on the roof of a sound proof box above the area of interest in each chamber. On the training day, mice were placed individually into the conditioning chamber for 6-minute acclimation, followed by three paired presentations of a clicker (CS) and footshock (US). A clicker (30 s, 4 Hz, 80 dB) presented through a speaker initiated each trial. The clicker co-terminated with an electric footshock onset (2 s, 0.5 mA) delivered through the grid floor. The inter-trial interval was 2 min. After the final clicker/shock pairing, the mice remained in the chamber for an additional 2 min without clicker or shock stimuli. The chambers were cleaned with 70% alcohol between each training session. Contextual and cued tests were performed at one month after fear conditioning using the method described previously (Barnes and Good, 2005). A video-tracking system EthoVision XT7 (Noldus, Wageningen, The Netherlands) was used for monitoring and recording. A freezing response (i.e., absence of movement except respiratory movement) was measured during the initial 6 min (pre-shock, free exploring) and the following 6 min (fear conditioning, US and CS paring) of training session as well as contextual and cued test sessions, respectively. Percentage of freezing duration in each session was analyzed by one-way ANOVA followed by Bonferroni multiple comparison post-test in Prism 5.00 (GraphPad Software Inc., California, USA). $P<0.05$ was considered as statistically significant. Behavioral data were presented as mean ± standard deviation.

2.3. ^{1}H MRS acquisition

Single voxel ^{1}H MRS experiments were acquired on a 7 T MRI (70/16 PharmaScan, Bruker Biospin GmbH, Germany) using a 23-mm birdcage quadrature RF coil for both transmitting and receiving. Three T2-weighted (T2W) scout images were first acquired with a rapid acquisition relaxation enhanced (RARE) sequence (TR/TE=4200/36 ms, RARE factor=8, spatial resolution=$0.109\times0.109\times0.48$ mm^3) for the localization of the voxel of interest (VOI). The size of VOI in the left hippocampus, the cingulate cortex and the left thalamus was $1.2\times2.5\times1.6$ mm^3, $1.2\times1.5\times2.5$ mm^3 and $2\times2\times2$ mm^3, respectively. After first- and second-order localized shimming with a FieldMap based procedure (Miyasaka et al., 2006), a full-width half-maximum linewidth of water signal of ≤15 Hz was achieved. The water signal was suppressed by variable RF pulses with optimized relaxation delays (VAPOR). A point-resolved spectroscopy (PRESS) sequence combined with outer volume suppression (OVS) was used for spectrum acquisition using TR/TE=2500/17 ms, spectral bandwidth=3 kHz, 2048 data points and 256 averages.

2.4. ^{1}H MRS spectral analysis

MR spectra were processed using the jMRUI software (http://www.mrui.uab.es/mrui/) as previously described (Chan et al., 2009). In brief, the raw data was apodized with a 15-Hz Gaussian filter and phase corrected. The residual water signal was filtered out using the Hackel–Lanczos singular value decomposition (HLSVD) algorithm. Chemical shifts of peaks were assigned with reference to the CH_3-group of NAA at 2.02 ppm. Metabolite area under the peak was quantified by quantum estimation (QUEST) method with subtraction approach for background modeling. The metabolite parameters were decorrelated from the background with truncation of initial data points given that macromolecules and lipids signals decay rapidly in time-domain. The numerical time-domain model functions of twelve metabolites, including alanine (Ala), aspartate (Asp), choline (Cho), total creatine (Cr), γ-aminobutyrate (GABA), glutamate (Glu), glutamine (Gln), glycine (Gly), lactate (Lac), myo-inositol (m-Ins), NAA and taurine (Tau), were used as prior knowledge in QUEST. These metabolite model signals were quantum mechanically simulated in NMR spectra calculation using operators (NMR-SCOPE) for the *in vivo* experimental protocol. Errors in measurement of noise and inadequate modeling of the overlapping background signal were calculated by the Cramér–Rao lower bounds (CRLBs), which were used to assess the reliability of metabolite quantitation. The quantification was considered as relevant only when the corresponding bound was below 25%. Cr was used as the internal spectral reference. Differences of NAA/Cr, Cho/Cr, Glu/Cr, Lac/Cr, m-Ins/Cr and Tau/Cr ratios at different time-points were statistically evaluated using repeated measures ANOVA test followed by Tukey's multiple comparison post-test with $P<0.05$ considered as significant. All data were presented as mean ± standard deviation.

3. Results

Fig. 1 shows the freezing responses measured during the initial 6-minute period (pre-shock) and the following 6-minute period (fear conditioning) of training session as well as contextual and cued test sessions, respectively. Significantly enhanced freezing responses ($P<0.05$) were observed during the 6-minute fear conditioning period compared to the 6-minute pre-shock period for acclimation, indicating that all mice quickly acquired fear memory. Subsequent contextual and cued tests showed the

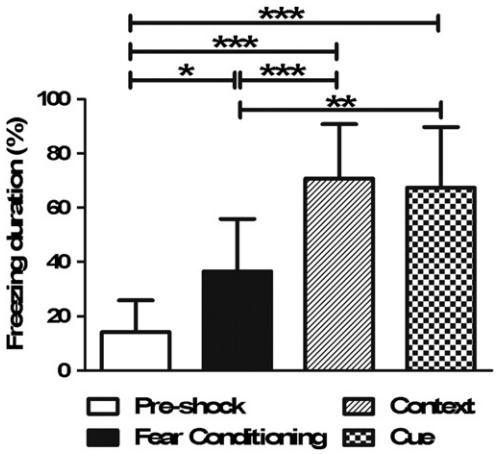

Fig. 1. Freezing responses measured during the initial 6 min (pre-shock, free exploring) and the following 6 min (fear conditioning) of training session as well as contextual and cued test sessions, respectively. One-way ANOVA followed by Bonferroni multiple comparison post-test was performed with $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data were presented as mean ± standard deviation.