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<th>Magnetic resonance spectroscopy reveals N-acetylaspartate reduction in hippocampus and cingulate cortex after fear conditioning</th>
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<td>Zhou, IY; Ding, AY; Li, Q; McAlonan, GM; Wu, EX</td>
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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 (1H MRS) of the hippocampus, cingulate cortex and thalamus of adult male C57BL/6N 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 1H 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.
Magnetic resonance spectroscopy reveals
N-acetylaspartate reduction in hippocampus and
cingulate cortex after fear conditioning

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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 \textit{ex vivo} while progressive ‘real-time’ changes \textit{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/6N 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.

\textbf{Keywords:} Proton magnetic resonance spectroscopy; fear conditioning; N-acetylaspartate; mouse brain; hippocampus; cingulate cortex; post-traumatic stress disorder; stress.
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; Schuff et al., 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 pairings, the CS alone comes to elicit physiological and behavioral fear reactions. Because fear 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/6N mice (N = 12) weighing 23-28g were used in this study. Animals were housed in groups of four under a 12:12 hour 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 (25×25×25 cm) 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 3 paired presentations of a clicker (CS) and footshock (US). A clicker (30 sec, 4Hz, 80 dB) presented through a speaker initiated each trial. The clicker co-terminated with an electric footshock onset (2 sec, 0.5 mA) delivered through the grid floor. The inter-trial interval was 2 minutes. After the final clicker/shock pairing, the mice remained in the chamber for an additional 2 minutes 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 minutes (pre-shock, free exploring) and the following 6 minutes (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 were 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 \( ^1H \) MRS acquisition

Single voxel \( ^1H \) 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 = 0.109×0.109×0.48 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 1.2×2.5×1.6 mm\(^3\), 1.2×1.5×2.5 mm\(^3\) and 2×2×2 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 \( \leq 15 \) 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 ¹H 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 CH₃-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 freezing duration significantly increased compared to the pre-shock period ($P < 0.001$ for both comparisons). These behavioral results confirm the long-term fear memories have been established successfully one month after fear conditioning. In addition, statistical evaluation reveals the freezing responses during the contextual and cued tests were also significantly elevated with regard to the fear conditioning period of the training session with $P < 0.001$ and $P < 0.01$, respectively. This finding of exaggerated trauma-related fear memories in mice suggests the development of PTSD-like symptoms.

Fig. 2 illustrates the typical localization of the VOIs in the left hippocampus, the cingulate cortex and the left thalamus (solid-line boxes) during $^1$H MRS experiment. The VOIs were positioned based on T2-weighted images of coronal and axial planes. Fig. 3 shows the representative raw $^1$H MRS spectra with QUEST fitting within each VOI from the same mouse before fear conditioning. Table 1 compares the mean metabolite to Cr ratios from all animals ($N = 12$) at each time-point in the left hippocampus, cingulate
cortex and the left thalamus, respectively. The average CRLBs of Cho, Glu, m-Ins, NAA and Tau were less than 15%, indicating reliable peak quantitation of these metabolites. In the hippocampus, significant reduction of NAA/Cr was observed at 1 day post-conditioning experiment ($P < 0.001$) compared to pre-conditioning. At 1 week post-conditioning, NAA/Cr was still significantly lower ($P < 0.05$). Cho/Cr decreased significantly ($P < 0.01$) at 1 day post-conditioning, but not at 1 week later. In the cingulate cortex, significant reduction in NAA/Cr ($P < 0.01$) was observed at 1 day post-conditioning. In contrast, no significant changes of metabolites were found in the thalamus except for decreased Glu/Cr ($P < 0.05$) at 1 day after the fear-conditioning.

4. Discussion
This study investigates the neurochemical alterations elicited by fear conditioning in the hippocampus, cingulate cortex and thalamus using in vivo $^1$H MRS. It is generally believed that the hippocampus, as part of its functions in spatial or configural information processing, is involved in contextual fear conditioning (Anagnostaras et al., 2001; Sanders et al., 2003). In behavioral studies, hippocampal lesions indicate that the hippocampus plays an important role in mediating the acquisition and consolidation of memory for the conditioning context (Phillips and LeDoux, 1992; Maren et al., 1997; Quinn et al., 2002; Maren and Holt, 2004). In rats, the medial prefrontal cortex consists of four main divisions, which from dorsal to ventral are the medial agranular, anterior cingulate, prelimbic, and infralimbic cortex (Vertes, 2006). The cingulate cortex is a region of the limbic system reciprocally connected to the hippocampus (Hamner et al., 1999). In rodents, the cingulate cortex is located in the dorsal portion of medial prefrontal cortex. Early studies suggested a key function of cingulate cortex in modulating
conditioned fear responses (Morgan and LeDoux, 1995). Therefore, as the key components of the fear circuitry, hippocampus and cingulate cortex often exhibit functional abnormalities in the in the anxiety disorders (Shin and Liberzon, 2010). Consistent with this, neurochemical changes in hippocampus and cingulate cortex are also characteristic of PTSD (Karl and Werner, 2010). The thalamus, whose function in the neurocircuitry of fear conditioning is to relay sensory inputs to amygdala and cortex (LeDoux, 2000), was also examined in this study. Metabolic changes induced by fear conditioning in these regions would not only provide neurobiological information related to learning and memory, but also give insights into the pathological mechanisms of neuropsychiatric disorders such as PTSD.

NAA, the major peak seen in $^1$H MRS spectrum, is a marker of neuronal density, integrity and health (Birken and Oldendorf, 1989). Regional reduction in NAA potentially indicates diminished neuronal density, neuronal loss or partially reversible cellular dysfunction (Malisza et al., 1998; Choi et al., 2007). Our findings of significantly reduced hippocampal NAA/Cr at 1 day post-conditioning could be explained by neuronal dysfunction at 1 day post-conditioning. Previous studies of patients with multiple sclerosis (De Stefano et al., 1995), focal ischemia (Higuchi et al., 1996) and schizophrenia (Deicken et al., 1999) showed that transitory NAA decrease can reflect a decrease in neuronal viability rather than neuronal loss. In the present study, the reversible hippocampal dysfunction might be a result of acute footshock stress. Stressful events, such as inescapable electric footshock, are known to cause deficits in hippocampal function (Rasmussen et al., 2002; Xi et al., 2011). More importantly, it is possible that the hippocampal dysfunction induced by acute stress may affect initial
encoding of contextual information associated with a traumatic experience. This could possibly underlie our observation of exaggerated responses during the contextual and cued test sessions at one month after fear conditioning. Such behavioral deficits in identifying safe contexts and explicit memory difficulties are phenomena paralleled between fear conditioning and PTSD (Rauch et al., 2006). Meanwhile, significantly reduced hippocampal NAA/Cr at 1 week post-conditioning possibly indicates reduced neuronal integrity and/or neuronal dysfunction. Previous $^1$H MRS studies of PTSD patients showed NAA decrease in the hippocampus with or without volumetric reduction, suggesting decreased hippocampal neuronal integrity (Villarreal et al., 2002; Schuff et al., 2008). Recently, investigation of a mouse model of PTSD by means of Manganese-enhanced MRI demonstrated that traumatic experience lead to volume loss in the hippocampus, possibly due to shrinkage of axonal protrusions (Golub et al., 2011). By applying the similar stimulus to theirs, our results of hippocampal NAA decrease may therefore reflect reduced neuronal integrity and contribute to trauma-related PTSD-like symptoms. Moreover, hippocampal cell dysfunction may also contribute to the decreased NAA level at 1 week post-conditioning. Functional imaging studies showed diminished activation of hippocampus in PTSD patients compared to healthy controls during explicit learning paradigms (Bremner et al., 2003; Kim and Jung, 2006).

NAA decrease was reported in the cingulate cortex of abused children with PTSD (De Bellis et al., 2000; Schuff et al., 2008). Moreover, functional imaging studies provide additional evidence for functional deficiency of anterior cingulate cortex in PTSD patients (Bremner et al., 1999; Lanius et al., 2001). However, our findings of decreased NAA/Cr at 1 day post-conditioning in cingulate cortex may mainly arise from neuronal
dysfunction induced by acute stress because the NAA level was normalized at 1 week post-conditioning. Previous study on a rat model of depression demonstrated acute stress inhibits long-term potentiation (LTP) at synapses and causes anterior cingulate cortex dysfunction (Rocher et al., 2004). In the present study, NAA/Cr in thalamus remained unchanged, possibly because thalamus is merely to relay sensory inputs during fear conditioning. This also indicates that the NAA changes were specific to hippocampus and cingulate cortex.

Cho is involved in phospholipid metabolism and thus acts as a marker for cellular membrane turnover (Podo, 1999). In this study, decreased hippocampal Cho/Cr at 1 day post-conditioning but not 1 week later may be associated with the acute traumatic stress induced by the electrical footshock. Cho decrease can be a result of reduced phospholipase C activity and protein synthesis in the hippocampus after inescapable shock (Dwivedi et al., 2005). Previous studies of stress models have shown consistent Cho level reduction (Czeh et al., 2001; Hong et al., 2009). However, its role in PTSD is controversial as both decreased and increased Cho have been reported in clinical studies (Karl and Werner, 2010).

Glu is the major excitatory neurotransmitter in the mammalian central nervous system. It is recognized as a potential excitotoxin and decreased levels have been found in many neurodegenerative conditions (Doble, 1999; Rego and Oliveira, 2003). However, the Glu decrease detected in thalamus at 1 day post-conditioning is transient since this change was not seen at 1 week later. Besides, decrease of NAA, the neuronal marker, was not observed and therefore the Glu decrease might not be related to neuronal degeneration. Though the concentration of Glu in brain is high, the Glu signal detected by $^{1}$H MRS is
low (de Graaf et al., 1991; Kauppinen and Williams, 1991). A peak overlap with other metabolites further limits the accuracy of Glu quantification. Therefore, the findings of Glu/Cr decrease in thalamus reported here should be regarded as preliminary and needs further investigation with the help of postmortem examination.

One limitation to the current study is the lack of measurement of absolute concentration. The NAA/Cr decrease in hippocampus and cingulate cortex reported here might imply Cr increase. Nevertheless, the reduction was not observed for other metabolites normalized to Cr or in thalamus. Note that such ratio analysis has been employed in many 1H MRS studies of PTSD, yielding consistent results. Another limitation is that the amygdala, a critical structure in fear conditioning, was not examined. The small size of amygdala in mouse makes it difficult to acquire reliable spectrum with sufficient SNR to detect metabolic changes. As metabolic changes following fear conditioning would certainly be predicted in this region, future study will be carried out with more efficient sequences such as chemical shift imaging (CSI). In the current study, only the left hippocampus was examined. According to the previous work by Siegmund et al. (Siegmund et al., 2009), the left hippocampal NAA level could be used to predict the susceptibility to trauma which the right hippocampal NAA level was only partially predictive. Moreover, the lateralized decrease of hippocampal NAA was also observed in human with PTSD (Mohanakrishnan Menon et al., 2003). Therefore, further investigation of NAA change in the right hippocampus with comparison to that of left hippocampus should also be carried out.
In conclusion, this study investigates the longitudinal metabolic changes induced by fear conditioning in the hippocampus, cingulate cortex and thalamus using \textit{in vivo} $^1$H MRS. The major findings of significantly lower NAA level in the hippocampus at both 1 day and 1 week post-conditioning indicate reduced neuronal dysfunction and/or neuronal integrity, contributing to the trauma-related PTSD-like symptoms. Significant reduction of NAA was also observed in the cingulate cortex at 1 day post-conditioning but not at 1 week post-conditioning, possibly due to the transitory neuronal dysfunction induced by acute stress. The $^1$H MRS detection of ongoing neurochemical changes elicited by fear conditioning can shed light on the mechanisms of learning and memory. Moreover, as a valuable animal model of studying the pathological mechanisms of PTSD, MRS in conjunction with the fear conditioning paradigm in rodents will find much needed application to the search for causal mechanisms and novel interventions in patients with psychiatric disorders.

\textbf{Acknowledgement}

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References


Figure Captions

Fig. 1. Freezing responses measured during the initial 6 minutes (pre-shock, free exploring) and the following 6 minutes (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.

Fig. 2. Typical localization of voxel of interest (VOI) in the hippocampus (a), cingulate cortex (b) and thalamus (c) (solid-line boxes) on coronal and axial slices of T2-weighted images for proton magnetic resonance spectroscopy measurements (L-left; R-right; A-anterior; P-posterior). The size of VOI in the left hippocampus, the cingulate cortex and the left thalamus was 1.2×2.5×1.6 mm$^3$, 1.2×1.5×2.5 mm$^3$ and 2×2×2 mm$^3$, respectively.

Fig. 3. Representative raw spectra (black) along with QUEST fitting (red) of the VOIs in the hippocampus, cingulate cortex and thalamus, respectively. The spectra were acquired from the same mouse before fear conditioning. Residuals of QUEST quantitation are shown in the top entry. Abbreviations: NAA, N-acetylaspartate; Glu, glutamate; Cr, creatine; Cho, choline; Tau, taurine; m-Ins, myo-inositol.
Table 1. Metabolite to Cr ratios and corresponding Cramér-Rao lower bounds (CRLBs) at 1 day before, 1 day and 1 week after the fear conditioning experiment in the hippocampus, cingulate cortex and thalamus, respectively. Data from all animals studied (N = 12) were presented as mean ± standard deviation (SD).

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<tr>
<th>Metabolites</th>
<th>Pre-conditioning (-1 day)</th>
<th>Post-conditioning (+1 day)</th>
<th>Post-conditioning (+1 week)</th>
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<td></td>
<td>Metabolite/Cr</td>
<td>CRLB (%)</td>
<td>Metabolite/Cr</td>
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<td><strong>Hippocampus</strong></td>
<td></td>
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<tr>
<td>Cho</td>
<td>0.30 ± 0.08</td>
<td>11.27 ± 2.95</td>
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<tr>
<td>Glu</td>
<td>0.84 ± 0.28</td>
<td>8.98 ± 2.36</td>
<td>1.00 ± 0.27</td>
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<tr>
<td>m-Ins</td>
<td>0.61 ± 0.14</td>
<td>8.26 ± 1.59</td>
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<tr>
<td>NAA</td>
<td>1.32 ± 0.30</td>
<td>7.19 ± 1.58</td>
<td>0.83 ± 0.21††</td>
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<tr>
<td>Tau</td>
<td>1.06 ± 0.35</td>
<td>5.94 ± 1.41</td>
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<td><strong>Cingulate Cortex</strong></td>
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<tr>
<td>Cho</td>
<td>0.33 ± 0.05</td>
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<td>Glu</td>
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<td>Tau</td>
<td>1.50 ± 0.33</td>
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<td><strong>Thalamus</strong></td>
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<tr>
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Repeated measures ANOVA were performed among three time-points with †p<0.05, ††p<0.01 and †††p<0.001 compared to pre-conditioning.
Figure(2)

(a) Hippocampus

(b) Cingulate Cortex

(c) Thalamus