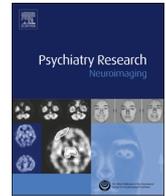




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Effects of fluoxetine on the amygdala and the hippocampus after administration of a single prolonged stress to male Wistar rats: In vivo proton magnetic resonance spectroscopy findings

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ABSTRACT

Posttraumatic stress disorder (PTSD) is an anxiety- and memory-based disorder. The hippocampus and amygdala are key areas in mood regulation. Fluoxetine was found to improve the anxiety-related symptoms of PTSD patients. However, little work has directly examined the effects of fluoxetine on the hippocampus and the amygdala. In the present study, male Wistar rats received fluoxetine or vehicle after exposure to a single prolonged stress (SPS), an animal model of PTSD. In vivo proton magnetic resonance spectroscopy (¹H-MRS) was performed –1, 1, 4, 7 and 14 days after SPS to examine the effects of fluoxetine on neurometabolite changes in amygdala, hippocampus and thalamus. SPS increased the *N*-acetylaspartate (NAA)/creatinine (Cr) and choline moieties (Cho)/Cr ratios in the bilateral amygdala on day 4, decreased the NAA/Cr ratio in the left hippocampus on day 1, and increased both ratios in the right hippocampus on day 14. But no significant change was found in the thalamus. Fluoxetine treatment corrected the SPS increases in the NAA/Cr and Cho/Cr levels in the amygdala on day 4 and in the hippocampus on day 14, but it failed to normalise SPS-associated decreases in NAA/Cr levels in the left hippocampus on day 1. These results suggested that metabolic abnormalities in the amygdala and the hippocampus were involved in SPS, and different effects of fluoxetine in correcting SPS-induced neurometabolite changes among the three areas. These findings have implications for fluoxetine treatment in PTSD.

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1. Introduction

Exposure to a stressful or traumatic event, such as combat, an earthquake or violence (Adami et al., 2006; Liberzon and Martis, 2006), may cause individuals to develop a stress-related cognitive and emotional disorder known as posttraumatic stress disorder (PTSD). The symptoms of this disorder include re-experiencing the trauma, avoidance, negative alteration in cognitions or mood, and altered arousal. Neuroendocrine studies have shown that PTSD patients have low levels of circulating cortisol because enhanced negative feedback occurs in their hypothalamus–pituitary–adrenal (HPA) axis (Liberzon et al., 1997, 1999; Birmes et al., 2000). Neuroimaging studies have revealed that PTSD patients have altered activity in the hippocampus and amygdala, two key areas in mood and HPA-axis regulation (Rauch and Shin, 1997; Hull, 2002; Shin et al., 2005; Hughes and Shin, 2011).

Using animal models to investigate the pathogenesis of PTSD has the potential to enhance understanding of this disorder and to provide information about the potential efficacy of drugs that can be used for its treatment. In recent years, the pathophysiology and behavioral characteristics of PTSD have been mimicked in several animal models. These models were based on exposure of animals to electric shock (Shimizu et al., 2006; Wakizono et al., 2007; Siegmund and Wotjak, 2007a, 2007b), inescapable shock (Rau et al., 2005), underwater trauma (Cohen et al., 2004) and predator-related cues (Adamec et al., 2006; Cohen et al., 2007). Each of these animal models showed aspects of validity for PTSD. The single prolonged stress (SPS) model, which was proposed by Liberzon et al. (1997, 1999) has been widely used. During SPS, animals are restrained for 2 h, subjected to a 20-min forced swim in 20–24 °C water, and exposed to ether anesthesia. Rats exposed to SPS express enhanced negative feedback in the HPA axis and low plasma levels of corticosterone, a phenotype that resembles the neuroendocrine features of PTSD in humans (Liberzon et al., 1997, 1999). Collectively, this evidence supports the validity of the SPS model. A number of behavioral studies showed that SPS-exposed rats experienced an increase in fear response and an

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impairment in memory (Imanaka et al., 2006; Takahashi et al., 2006; Iwamoto et al., 2007; Kohda et al., 2007), which reveals hippocampal and amygdalar dysfunctions that mimic the clinical symptoms of PTSD. In the neurocircuitry of fear conditioning, the thalamus relays sensory inputs to the amygdala (LeDoux, 2000). However, the literature on the effect of SPS on the thalamus is limited. Therefore, the thalamus was also chosen as a region of interest. Selective serotonin reuptake inhibitors (SSRIs) are widely used to treat psychiatric disorders (Fichtner et al., 1994; Ballenger et al., 2000, 2004). Because SSRIs inhibit the reuptake of serotonin (5-HT), only acute administration of these drugs causes increased 5-HT concentrations in the amygdala and the hippocampus (Parks et al., 1998; Ramboz et al., 1998). In the past 30 years, several studies have examined the effects of SSRIs on emotion and on the different stages of fear-motivated learning (Davidson et al., 1990, 2001; March, 1992). The SSRI fluoxetine is recommended as a first-line drug treatment for PTSD. Fluoxetine is effective in improving the numbing and hyperarousal symptoms that PTSD patients experience (Martenyi et al., 2002; Martenyi and Soldatenkova 2006). However, little work has directly examined the effects of fluoxetine on the hippocampus and amygdala, respectively, particularly metabolic effects.

This study investigated the effects of fluoxetine in the SPS animal model to determine any neurochemical changes that might occur in the amygdala, hippocampus and thalamus using *in vivo* proton magnetic resonance spectroscopy ($^1\text{H-MRS}$). $^1\text{H-MRS}$ estimates metabolic changes in the living brain by measuring the concentrations of *N*-acetylaspartate (NAA), creatine (Cr) and choline moieties (Cho). NAA is only expressed in neurons and not in glial cells (De Stefano et al., 1995). Decreased NAA is indicative of neuronal death, decreases in neural density (Martin et al., 2001), and abnormal metabolic processes (Tamiya et al., 2000). Cr is a measure of cellular energy metabolism and is usually taken as the standard denominator in MRS ratio analyses. Cho is highly expressed in glial cells, and changes in Cho level reflect changes in the number, size, and density of glial cells. Our previous study found an increased apoptosis rate and abnormal ultrastructure in the hippocampus and amygdala (Ding et al., 2010; Li et al., 2010). Thus, by measuring levels of NAA and Cho, neuronal and glial cell integrity in the brain can be assessed (Hammen et al., 2003; Sijens et al., 2005). Previous magnetic resonance imaging (MRI) studies revealed that PTSD patients have altered function as well as metabolism in various brain regions, and these abnormalities have been reversed after SSRI treatment (Shin et al., 2005; Etkin and Wager, 2007; Morey et al., 2009; Murrough et al., 2011; Zhou et al., 2012; Xiong et al., 2013). However, these studies were limited because the effects of fluoxetine were monitored at certain time points following the onset of PTSD but not throughout the course of treatment. Thus, these studies do not provide data collected at multiple time points to fully evaluate the efficacy of fluoxetine.

In the present study, we used male Wistar rats treated with fluoxetine or vehicle after exposure to a single prolonged stressor (SPS). *In vivo* proton MRS was performed -1 , 1 , 4 , 7 and 14 days after SPS to examine longitudinal effects of fluoxetine on neuro-metabolite levels in the amygdala, hippocampus and thalamus.

2. Methods

2.1. Animals

Male Wistar rats (8–10 weeks old, 220–250 g) were housed in a colony room that was maintained at $22 \pm 1^\circ\text{C}$ (mean \pm SEM) on a 12/12-h light/dark cycle. The rats were provided food and water *ad libitum*. All experiments were approved by the Ethics Committee of China Medical University and conducted in accordance with the Guideline Principles on Animal Experimentations for Laboratory Animal Science (China Medical University).

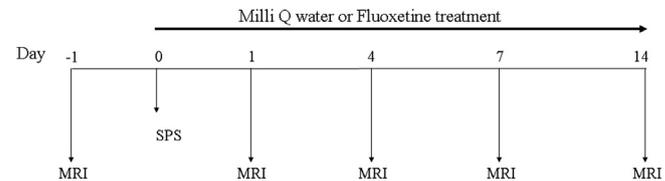


Fig. 1. The time schedule of the procedure is illustrated. The numbers indicate the day. Rats were treated immediately with fluoxetine or MilliQ water for 14 days after the rats were exposed to SPS stimulation. MRI examinations were performed 1 day before SPS (-1 day) and 1, 4, 7 and 14 days after SPS exposure.

2.2. Experimental design

Rats ($n=60$) were randomly assigned to the following four groups: rats not subjected to SPS followed by vehicle administration, the “non-SPS+vehicle group”; rats not subjected to SPS followed by fluoxetine administration, the “non-SPS+fluoxetine group”; rats subjected to SPS stimulation followed by vehicle administration, the “SPS+vehicle group”; and rats subjected to SPS stimulation followed by fluoxetine administration, the “SPS+fluoxetine group”. Milli-Q water was available freely for the non-SPS+vehicle and SPS+vehicle groups. Fluoxetine (Pantheon, France, 120 mg/l) dissolved in Milli-Q water was available freely to the non-SPS+fluoxetine and SPS+fluoxetine groups. The non-SPS+vehicle and SPS+fluoxetine groups were exposed to SPS, and then the SPS+fluoxetine group was immediately treated with fluoxetine for 14 days. This regimen was chosen based on previous studies (Karpova et al., 2011; Camp et al., 2012) to provide an average self-administered daily dose of 10 mg/kg. Fluoxetine and water consumption were measured from bottle graduations and converted to a daily dose per mg/kg body weight. Oral intake of fluoxetine mimicked its clinical use and prevented the stress associated with injections. $^1\text{H-MRS}$ measurements were performed 1 day before SPS exposure (-1 day) and 1, 4, 7 and 14 days after SPS exposure. Fig. 1 illustrates the experimental procedure.

2.3. Single prolonged stress (SPS) procedure

The SPS procedure developed by Liberzon et al. (1997) was used in this study. Rats were restrained for 2 h and then immediately forced to swim for 20 min in water ($20\text{--}24^\circ\text{C}$) that was in a plastic tub (55.6 cm diameter, 45.4 cm height) and filled two-thirds from the bottom. After a 15-min recuperation period, rats were exposed to ether (using a desiccator) until general anesthesia occurred, defined as loss of toe and tail pinch responses (< 5 min). Immediately after the induction of general anesthesia, rats were removed and placed in their home cages. In the present study, we performed behavioral tests (data not shown) to examine whether the animals developed PTSD after SPS exposure. Rats without PTSD development were not used in the present study.

2.4. $^1\text{H-MRS}$ acquisition and spectral analysis

All MRI experiments were conducted in a horizontal bore animal scanner (7 T Bruker Biospec USR 70/30; Bruker Biospin GmbH, Germany). Anesthesia was induced by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (80 mg/kg) (Kumar et al., 2013). Animals (both vehicle and fluoxetine groups) were placed in a prone position on an animal bed and then slid into the center of the magnet bore. Radiofrequency (RF) excitation was accomplished with a 72-mm inner diameter (ID) linear birdcage coil, and a 30-mm receiver-only surface coil was used for signal reception. Localized proton spectra were acquired using a point-resolved spectroscopy (PRESS) sequence. Voxels of $2\text{ mm} \times 2\text{ mm} \times 2\text{ mm}$, $2\text{ mm} \times 3\text{ mm} \times 3\text{ mm}$, and $3\text{ mm} \times 3\text{ mm} \times 3\text{ mm}$ were placed within the hippocampus, amygdala complex, and thalamus, respectively. The maximum volume contained tissue from the intended structures, minimizing the contribution from the surrounding tissue and partial volume effects. First and second order localized voxel shimming was performed with a fast, automatic shimming technique by mapping along projections, which resulted in a full-width half-maximum line width of ≤ 15 Hz for the water signal. The PRESS spectra were obtained with the following scan parameters: repetition time/echo time = 2500/17 ms, spectral bandwidth = 3 kHz, 2048 data points, and 256 averages.

All spectra were initially processed using the scanner's Topspin 2.1 software (Bruker Biospin, Germany). Brain metabolites were quantitatively assessed by analyzing the raw MRS data utilizing an LC model (Provencher, 2001). The LC model automatically calculated the metabolite concentrations and the uncertainties using the Cramér-Rao lower bound (CRLB) formalism (Provencher, 2001). An estimate was relevant when the corresponding bound was below 20%. Relative concentrations of all the observed metabolites and macromolecules were calculated for further analysis. Spectra were baseline-corrected using an average of the spectral regions from 0 to 2 ppm. NAA, Cr and Cho were quantified by fitting the peaks to a Gaussian line shape using the central 50% of the resonance at 2.02 ppm. Using this technique, resonances were overlapped with other resonances that were

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