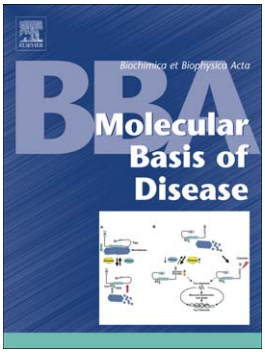



# Specific patterns of spinal metabolites underlying $\alpha$-Me-5-HT-evoked pruritus compared with histamine and capsaicin assessed by proton nuclear magnetic resonance spectroscopy

Taotao Liu, Zhigang He, Xuebi Tian, Ghulam Mustafa Kamal, Zhixiao Li, Zeyuan Liu, Huili Liu, Fuqiang Xu, Jie Wang, Hongbing Xiang

PII: S0925-4439(17)30098-4
DOI: doi:10.1016/j.bbadis.2017.03.011
Reference: BBADIS 64717

To appear in: *BBA - Molecular Basis of Disease*

Received date: 12 April 2016
Revised date: 22 March 2017
Accepted date: 22 March 2017

Please cite this article as: Taotao Liu, Zhigang He, Xuebi Tian, Ghulam Mustafa Kamal, Zhixiao Li, Zeyuan Liu, Huili Liu, Fuqiang Xu, Jie Wang, Hongbing Xiang, Specific patterns of spinal metabolites underlying $\alpha$-Me-5-HT-evoked pruritus compared with histamine and capsaicin assessed by proton nuclear magnetic resonance spectroscopy, *BBA - Molecular Basis of Disease* (2017), doi:10.1016/j.bbadis.2017.03.011

# Specific patterns of spinal metabolites underlying α-Me-5-HT-evoked pruritus compared with histamine and capsaicin assessed by proton nuclear magnetic resonance spectroscopy

Taotao Liu [a, c, 1]; Zhigang He [a, 1]; Xuebi Tian [a]; Ghulam Mustafa Kamal [b, d]; Zhixiao Li [a], Zeyuan Liu [e]; Huili Liu [b]; Fuqiang Xu [b, d]; Jie Wang [b, d, *]; Hongbing Xiang [a, *]

[a]: Department of Anesthesiology and Pain Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan , Hubei, 430030, P. R. China;

[b]: Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan, Hubei, 430071, P. R. China;

[c]: Department of Anesthesiology, Peking University Third Hospital, Beijing 100191, China.

[d]: University of Chinese Academy of Sciences, Beijing, 100049, P. R. China;

[e]: College of Life Science, Wuhan University, Wuhan, Hubei ,430076, P. R. China

[1] Equal contribution to the work

**Corresponding authors**:

**Jie Wang**: E-mail: jie.wang@wipm.ac.cn; Tel: +86-27-87187359; Fax: +86-27-87199543;

**Hongbing Xiang**: E-mail: xhbtj2004@163.com; Tel.: +86-27-83663173; Fax: +86-27-83662853.

**Abstract:** The mechanism behind itching is not well understood. Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopic analysis of spinal cord extracts provides a quick modality for evaluating the specific metabolic activity of α-Me-5-HT-evoked pruritus mice. In the current study, four groups of young adult male C57Bl/6 mice were investigated; one group treated with saline, while the other groups intradermally injected with α-Me-5-HT (histamine independent pruritogen), histamine (histamine dependent pruritogen) and capsaicin (algogenic substance), respectively. The intradermal microinjection of α-Me-5-HT and histamine resulted in a dramatic increase in the itch behavior. Furthermore, the results of NMR studies of the spinal cord extracts revealed that the metabolites show very different patterns for these different drugs, especially when comparing α-Me-5-HT and capsaicin. All the animals in the groups of α-Me-5-HT and capsaicin were completely separated using the metabolite parameters and principal component analysis. For α-Me-5-HT, the concentrations of glutamate, GABA, glycine and aspartate increased significantly, especially for GABA (increased 17.2%, $p$=0.008). Furthermore, the concentration of NAA increased, but there was no significant difference (increased 11.3%, $p$=0.191) compared to capsaicin (decreased 29.1%, $p$=0.002). Thus the application of magnetic resonance spectroscopy technique, coupled with statistical analysis, could further explain the mechanism behind itching evoked by α-Me-5-HT or other drugs. It can thus improve our understanding of itch pathophysiology and pharmacological therapies which may contribute to itch relief.

***Keywords****: Itch; Spinal cord; Metabolites; Nuclear magnetic resonance spectroscopy; GABA.*

## Introduction

Pruritus represents a major health concern as an unpleasant sensation accompanied by the characteristic scratch desire or reflex [1, 2]. It's widely acknowledged that the spinal cord is responsible for the generation and transduction of itch induced by many pruritogens. Several spinal theories have been proposed to explain the basic mechanism behind the itch, such as spinal neurons which express the G-protein coupled receptor Mrgpra 3, gastrin-releasing peptide receptor (GRPR), natriuretic polypeptide B (Nppb) and neuromedin B receptor (NMBR) are involved in the transmission of acute itch and long-lasting itch [3-7]. Furthermore, metabolomics approaches for the measurement of the local metabolites is a powerful tool when characterizing the pathologic and metabolic processes of central nervous system diseases [8-10]. A better understanding of the spinal metabolites underlying itch processing with specific linkage as to which neural processes relate to itch perception and scratching, is urgently needed in order to elucidate the itch mechanisms and develop new approaches of itch relief.

The development of nuclear magnetic resonance (NMR) methodologies over the past decades has revolutionized basic neurochemistry research. Proton NMR ($^{1}$H-NMR) is a basic technique for the measurement of regional metabolite concentrations in vitro or in vivo, such as γ-aminobutyric acid (GABA), glutamate (Glu), N-acetylaspartate (NAA), alanine (Ala), creatine (Cr), glutamine (Gln), lactate (Lac), etc [11, 12]. To our knowledge, NMR has not yet been used to assess itch-induced metabolic responses in the spinal cord.

In our previous research, the inhibition of itch-related responses was identified through selectively ablated serotonergic signals [2]. The purpose of the injection of alpha-methyl-5- hydroxytryptamine (α-Me-5-HT, a serotonin derivative) or histamine to animals was to construct experimental models for studying the mechanisms of pruritus, as the serotonergic system is involved in itch generation and processing [7, 13]. In order to pursue the specific patterns of metabolites changes in spinal cords of α-Me-5-HT, two different pruritogens (α-Me-5-HT and histamine) and one algogenic compound (capsaicin) were employed in the current study (Reviewer's valuable suggestion). We hypothesized that the concentrations of the important metabolites in the spinal cord, such as glutamate and GABA, should change with different kinds of phenotypes (itch and pain). $^{1}$H-NMR spectroscopy was employed to investigate the absolute concentrations and variations of the spinal metabolites during the processing of pruritis or pain produced by intradermal injection of α-Me-5-HT, histamine or capsaicin in the present study.

## Materials and methods

### Animals

Young adult male C57Bl/6 mice (6 weeks, 22-25g) were obtained from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All the animals were housed in the plastic cages (3 in each cage) and maintained on a 12-h light/dark (lights at 7:00 am-7:00 pm) cycle, with food and water available *ad libitum*. The behavioral experimental procedures were performed in

accordance with the protocols approved by the Animal Care and Use Committee of Tongji Medical College.

**Itch model and behavior test**

In order to explore the influence of the itch behavior on different metabolites on the spinal cord, the 35 mice were randomly divided into four groups: normal saline (NS, n=8) treated group, α-methyl-5-hydroxytryptamine (α-Me-5HT, Tocris, England; 30μg, 50μl, n=9) and histamine (Sigma-Aldrich, MO, USA; 500μg, 50μl, n=9) treated groups and capsaicin (Sigma-Aldrich, MO, USA; 40μg, 50μl, n=9) treated group.

The itch model and scratching/wipes behavior test were performed similar with previous researches [2, 4, 13, 14]. Briefly, under isoflurane (2% in pure oxygen) anesthesia, both the nape of the neck (approx. 1 cm circle) and the cheek (approx. 5 × 8 mm area) were shaved three days before the behavior test. Each mouse was habituated in separate plastic containers (9 × 9 ×13 cm) for 45 minutes every day for three successive days before the day of the experiment. On the 4th day, each mouse was habituated for 30 min, shortly removed from the container and quickly administered with an intradermal injection of 50μl α-Me-5HT, histamine, capsaicin or normal saline at the nape skin of the neck. To manifest the effects of capsaicin on the algogenic behavior, 14 more mice was used for the measurement. Capsaicin was intradermally injected into the cheek (n=4) or the nape of neck (n=6), while the control group (n=4) was treated with saline intradermal injection to the cheek. The mice were immediately shifted back to the container. Video

cameras were used to record the activities of the mice for a 30-min post injection period. The number of scratches or wipes was counted and the differences between the control and the experimental groups were obtained to verify the effect of the drugs.

**Spinal samples for NMR study**

For the itch related studies, the *c-fos*-like immunoreactivity was always investigated 2h after injection [15, 16], thus the changes of metabolites in the spinal cord of animals were detected two hours after the injection. In order to avoid the impact of post-mortem changes (Reviewer's suggestions), the anesthetized mice were microwaved (0.75kw, 15s) using a normal microwave machine (Midea, PJ21C-AU, China). The bottom half of the spinal cords (white color, $C_5$-$C_8$) were quickly removed from the vertebral cervicals to a 2ml EP tube. The tissue was weighed and immediately frozen. All the samples were maintained in a refrigerator at -80$^{o}$C until further processing.

HCl/methanol (80μL, 0.1M) was added to the frozen tissues. Then the tissues were homogenized with Tissuelyser for 1.5 min at a frequency of 20Hz (Tissuelyser II, QIAGEN, German). Another 400μL 60% ethanol (vol/vol) was added in the EP tubes and the mixture was homogenized again under the same conditions. The mixture was centrifuged at 14,000 g for 15 min and the supernatant was collected. The extraction steps were repeated twice with 1200μL 60% ethanol to extract the substance. All the supernatants were collected together and desiccated with the centrifugal drying apparatus (Thermo Scientific 2010, Germany). The dried product was collected for further NMR

studies.

*Preparing the samples for NMR detection:* Phosphate buffer [pH=7.2, 60μl, 120mg/L 3-(Trimethylsilyl) propionic - 2, 2, 3, 3, $d_4$ acid sodium salt (TSP, 269913-1G, Sigma-Aldrich) in $D_2O$] and 540 μL double distilled water were added to dissolve the dried product and TSP was set as the internal standard. The solution was mixed evenly with a high-speed vortex and centrifuged at 14,000 g for 15 min, and the supernatant (530μl) was withdrawn and transferred to a 5mm NMR tube for $^1$H-NMR analysis.

**NMR Spectroscopic Analysis**

All NMR spectra were acquired at 298 K on a BrukerAvance III 600 MHz NMR spectrometer equipped with an inverse cryogenic probe (BrukerBiospin, Germany). The $^1$H-NMR spectra were acquired with a standard WATERGATE pulse sequence [17]. The 90° pulse length was adjusted to about 10.1μs for each sample and 128 transients were collected into 32k data points over a spectral width of 20 ppm.

In order to assign the metabolites in the $^1$H-NMR spectra, a series of two-dimensional (2D) NMR spectra were collected for a random sample (Fig. S1). The 2D spectra include $^1$H-$^1$H correlation spectroscopy (COSY), J-resolved spectroscopy (JRES), $^1$H-$^1$H total correlation spectroscopy (TOCSY), $^1$H-$^{13}$C heteronuclear single quantum correlation (HSQC) and $^1$H-$^{13}$C heteronuclear multiple bond correlation (HMBC) 2D NMR spectra (An example of 2D NMR spectra analyses was added in the supplemental material). All the NMR studies were completed using the commercial

software TOPSPIN (V3.0, BrukerBiospin, Germany).

**NMR Data Processing**

The NMR spectral data processing was completed in the commercial software TOPSPIN and NMRSpec, a home-made tool using MATLAB code [18], which has been successfully used for several NMR studies [19, 20]. For $^{1}$H spectra, the experimental window function was employed with a line broadening factor of 1 Hz prior to the Fourier transformation. All the spectra were manually phased and baseline corrected in Topspin. The chemical shift of the spectra was adjusted with the internal standard TSP (δ=0). The peak alignment and integration were completed in NMRSpec. Most amino acids were located in the region of 1.1-4.2 ppm, and very few signals were there in other regionS or otherwise overlapped with macmolecular/lipids' signals. Similarly with the brain extracts [21], the spectral regions δ 1.1-4.2 ppm were collected. The basic theory of the peak alignment and integration has been described before [22]. The 25 related peak areas were calculated separately (Fig. 1, $Ace_2$ and $GABA_3$ combined together).

The weights of the tissues were very similar ~ around 10 mg and thus the $^{1}$H-NMR spectra could be normalized by the tissue weight: All the peak intensities of a spectrum could be normalized with the tissue weight to generate a normalized spectrum. Then all the normalized spectra in the same group were averaged point by point with the total number of animals in every group after the $^{1}$H-NMR spectra were aligned using NMRSpec. After that the average spectra was obtained.

The absolute concentrations (μmol/g wet weight) of the metabolites were calculated from the related peak areas in spectrum of the sample, the internal standard NMR spectra (TSP) and the weight of specimen. The procedure of calculation is given in the following equation:

$$C_{met} = \frac{A_{met}/(R_{met} * N_H)}{A_{TSP}} * (C_{TMSP} * V_{TMSP}) * 9/Wt \qquad (1)$$

Where $A_{met}$ and $A_{TSP}$ are the area of the related peaks of the target metabolites and TSP; $N_H$ is the number of protons of the metabolites within the certain area of $A_{met}$; $R_{met}$ is a constant value for a certain metabolites and was calculated from the ratio between the partial NMR signal of the standard metabolite between the selected region (almost pure chemical signal) in a real sample and the whole proton signal in the standard spectrum; $C_{TSP}$ and $V_{TSP}$ are the concentration and volume of TSP standard solution added in the NMR tube respectively; $W_t$ is the total wet weight of the specimen, and 9 is the number of protons in the TSP. Furthermore, as there are always many peaks for the same metabolites, therefore it was better to select a pure signal which was not overlapped by other signals, such as $Asp_3$, $Tau_1$, $Ala_3$, $Cre_1$, Choline and $Lac_3$, where $R_{met}$ was equal to 1. However, if there was no pure signal available for a related chemical, it was better to select part ($R_{met}$) of the related signal; such as $GABA_3$ (0.534), $Glu_4$ (0.871), $Myo_{1,3}$ (0.307), $Gln_4$ (0.392), and $NAA_2$ (0.587). In case, the signals were totally overlapped, then it was impossible to use them to calculate the absolute concentration.

**Statistical analysis**

Multivariate data analysis was performed with the software SPSS (Version 22) and home-made code NMRSpec in MATLAB [18].

During the sample processing, there was no easy way for the ethanol content to be removed completely. As scuh, the signals of ethanol could influence the spectral analysis in the related spectral regions (δ: 1.15-1.22 and 3.622-3.710), so the ethanol signals were discarded.

The principal component analysis (PCA) was conducted using the absolute concentration of the metabolites to generate an overview for group clustering and to search for the possible outliers.

One-way analyses of variance (ANOVA) were employed to determine the significance of differences for both behavior data and metabolite levels followed by least significant difference (LSD) post-hoc tests to examine differences among groups. The criterion for statistical significance was a probability value of 0.05. All results are presented as means ± SEM.

**Results**

**Evaluation of the behaviors induced by different stimulus**

The time course of the scratching/wipes behaviors was compared among the saline treated (control) group and drug-treated groups (α-Me-5-HT, histamine or capsaicin).

The number of scratches elicited by different stimuli is illustrated in Fig. 2A and 2B, which shows the time course of scratching behavior during the 30-min test period with 5-min intervals after the intradermal injection of α-Me-5-HT, histamine or normal saline in the nape of the neck. Compared with the control group, all the mice in the treated groups exhibited a dramatic increase in scratching behaviors per 5 min evoked by α-Me-5-HT or histamine (Fig. 2A). Injection of pruritic stimulation drugs induced many more ($F=68.751$, $p<0.001$) scratching responses during a period of 30 min (α-Me-5-HT, 266.6±23.10; histamine, 93.22±10.06) than in the control group (7±2.87) (Fig. 2B).

The time course of the scratching/wipes behaviors was compared among the saline (cheek treated) and capsaicin treated groups (cheek or nape of the neck treated). Compared to the control group, scratch-like behaviors (the hind paws of the treated mouse were used to slightly touch the injection site which was more likely a reflex for alleviating pain induced by the capsaicin instead of a true scratch behavior, as the forelimbs were not able to reach to the nape of the neck) were found to have increased dramatically after the capsaicin was treated into the nape of neck, while the wipes behaviors emerged and increased in the 5 minutes after capsaicin injected into the cheek (Fig. 2C). The total numbers of wipes or scratch-like behaviors during the 30-min test period was calculated in Fig. 2D ($F=14.266$, $p=0.001$). The difference between the capsaicin group (into nape or into cheek) and the NS group was significant ($p_{nape\text{-}NS}=0.003$, $p_{cheek\text{-}NS}=0.001$). Data is represented as Means± SEM. The asterisks denote significant differences between the two groups (**: $p<0.01$, ANOVA test, followed by LSD post-hoc tests).

**Impact of the microwave treatment on the metabolites**

In order to avoid the post-mortem changes of the metabolites (Lactate, GABA, alaine, *etc*) (Reviewer's suggestion) [23], the microwave method was employed in the current study to denature the enzymes in the tissue with a high temperature treatment. A normal household machine was used in the current study due to the commercial head focused microwave machine is too expensive [24]. The average tissue weight decreased to 10.44±0.44 mg (from 16.56 ± 0.76 mg without microwave), which was caused by water being lost during the period of microwave treatment. Although the tissue weight decreased, the lactate concentration dropped to 5.53 ± 1.28μmol/g (from 12.02 ± 0.93μmol/g without microwave). The results verified that the enzymes in the tissue were totally denatured after the microwave treatment and concentrations of the other metabolites could reflect the concentrations at the real time of treatment.

**Absolute concentration of the metabolites**

The average NMR spectrum of the extracts for the spine samples in the control group and the drug-treated groups (two pruritic stimulation related drugs: α-Me-5-HT and histamine; one algogenic stimulation drug: capsaicin) were calculated (Fig. 1), which was obtained from the normalization of the spectra by the weight of the tissues.

In order to pursue the effects of the drugs on the mice, differences of the average spectrum among the treated groups and control group were also calculated from the difference of two average spectra point by point (Fig. 3). For a clear visualization, the three different spectra were collected in the same figure. There is no standard deviation information in this figure, thus it could not be directly utilized to provide the effect of the drugs on the metabolites. However, it could roughly evaluate the tendency of results of these three different drugs. α-Me-5-HT and capsaicin have two different influence patterns on the changes of the metabolites - one positive and the other one negative - especially NAA, alanine, aspartate, GABA and glutamate (two major neurotransmitters).

In order to precisely assess the effects of these three different drugs, absolute concentrations of all the related metabolites were calculated and compared (Table S1 and Fig. 4). The raw data of average and standard deviation of some selected chemicals are displayed in Fig. 5. Statistical significance of differences among different groups was assessed by one-way ANOVA followed by LSD post-hoc multiple comparison test, and the results were collected in Table S1. The concentrations of most metabolites were significantly changed among the control and groups treated with α-Me-5-HT, histamine and capsaicin. In order to pursue the contribution of the metabolites on the drugs' treatment, metabolites in the drugs treated groups was detailed compared with the control group using the method of one-way ANOVA followed by LSD post-hoc multiple comparison test. The results were illustrated in Fig. 4.

The concentration of lactate was used to verify whether the microwave method could be used to cease metabolism in the animal tissue for a short period of time. It does not increase to a significantly higher value (remained around 12μmol/g even using higher wet tissue weight with fast operation under cold temperature). Thus the metabolic reaction had been totally ceased in the mice brain with the microwave method. Furthermore, there was also no significant difference among these four different groups with the ANOVA analysis.

From the results of the statistical analysis, most of the metabolites in the spinal cord had changed significantly after the drugs' injection, especially for α-Me-5-HT and capsaicin, such as glycine, glutamate, GABA, taurine and aspartate. It is interesting that the tendency of most changes was opposite in the itch and pain animal models. Similarly, in comparison with the control group, the chemical NAA did not increase significantly in the group treated with α-Me-5-HT but did decrease in the capsaicin-treated group.

***Results of PCA analysis***

Using the absolute concentration of the metabolites, PCA analysis was performed in SPSS to generate an overview for group clustering and to search for the possible outliers. The PCA method was used to reduce the dimensions of variables by dropping unnecessary data. At the end, the first three principle components PC1, PC2, and PC3 among the control and pruritic stimulation groups, control and algogenic stimulation models, and pruritic and algogenic stimulation models were collected. The total

contributions of the first three components were higher than 80% of the variance for all the variables and played major roles in all the comparison groups. The samples had a tendency to cluster in both of α-Me-5-HT and capsaicin treated groups (Fig. 6A and 6B) and the samples in both of the pruritic stimulation drugs were almost mixed together (Fig. 6C). However, the samples with α-Me-5-HT and capsaicin treated groups were completely separated (Fig. 6D), which means that these two drugs have significantly different effects on the animals and consistent with their effects on the animal behavior (pruritic and algogenic stimulation).

**Correlation coefficient of the metabolites:**

The whole correlation matrix of these spinal metabolites is shown in Table 1. In the current study, if the correlation coefficient of two metabolites was higher than 0.80, it could be considered that there was a strong correlation between the two, and might be with similar aspects of the functions encoded. The correlation coefficients of alanine, creatine or taurine with the other metabolites were negative, which were also verified by Fig. 3. Changes in these three metabolites tend to be similar to each other and opposite to the other metabolites. The concentrations of NAA, glutamate, glycine, myo-institol, GABA, and aspartate were positively correlated, especially between NAA and glutamate, myo-institol and NAA, aspartate and NAA, etc. All of these metabolites tended to change similarly after different kinds of drug stimulations.

## Discussion

This study delineated the changes of the metabolites among the spinal metabolites during α-Me-5-HT-induced pruritic behavior as assessed by $^{1}$H-NMR. In order to verify if the recognized neurotransmitters are itch-specific, two other drugs were added: one was histamine for pruritic stimulation under histamine dependent mechanism; the other was capsaicin for algogenic stimulation. The principal findings were as follows: (1) intradermal microinjection of α-Me-5-HT resulted in a dramatic increase in itch-related behavior; (2) itch stimulation by α-Me-5-HT significantly increased the transmitters of glutamate, GABA, glycine and aspartate in spinal cord, and decreased taurine; (3) changes in most of the neurotransmitters were almost opposite between pruritic and algogenic stimulation groups, and also different between two pruritic stimulation drugs, which means that the animal behaviors were due to the influence of different mechanisms.

### *Application of NMR-method*

There is a consensus that $^{1}$H-NMR is a sensitive method for the analysis of biological tissues in vivo and in vitro [10, 25, 26], as it provides more biochemical information on acute and chronic pathological processes than conventional MRI. The application of $^{1}$H-NMR in pain analysis has been widely documented [11, 12, 27], which indicates that there exists the feasibility of this technique in detecting metabolic changes

during the pathological pain conditions. A large number of neuroanatomical, neurophysiologic and neurochemical mechanisms are thought to contribute to the generation and transduction of itch [28-30]. The spinal cord is a critical area associated with itch processing and modulation [4, 5, 30]. However, the spinal metabolomics responsible for itch has not been completely delineated. To explore the spinal mechanisms underlying specific itch, the behavioral responses (scratching) and spinal metabolites produced by injection of α-Me-5-HT and histamine were assessed. Furthermore, the effects of another algogenic stimulant drug, capsaicin, on the spinal metabolites were also measured. The results indicated that NAA, GABA, glutamate, alanine, taurine, creatine, choline, myo-inositol, aspartate, glutamine, glycine and lactate resonances were detected in spinal tissues.

*Influence of the post injection period*

According to reviewer's suggestion, another groups with different treatment period (10min) were further added to compare the influence of the drugs on the metabolites, as the maximum number of animal behavior of scratching/wipes would always appear around 10min.

With the same protocol, the NMR spectra of the samples from animals with 10min post-injection treatment were collected and the comparison of the metabolites with the control group were conducted and shown in Table S2. The changes of the metabolites were very similar between two different periods for the itch related drugs, such as the

increase of glutamate, GABA, aspartate and NAA for α-Me-5-HT, and the increase of alanine for histamine (Table S2). Almost all the metabolites in the group of histamine treated did not change at all in both periods. Furthermore, there are several differences between 10min and 2h groups for α-Me-5-HT treated group, such as creatine and taurine. The metabolites in the group of 10 min's capsaicin-treated group had changed only slightly, however, most of the metabolites in the group of 2h post-treated with capsaicin had significantly changed. Thus, the relationship between the metabolites and algogenic behavior caused by capsaicin is more complicated, and needs further investigation. Here the relationship between different metabolites and itch related behavior was discussed.

*Metabolites*

Compared to the control and itch stimulation groups, the mice in α-Me-5-HT treated group displayed a dramatic increase in the level of glutamate, GABA, glycine, and aspartate, as well as a decrease in creatine and taurine in the $C_5$-$C_8$ regions of the spinal cord. The concentration of NAA also increased after α-Me-5-HT stimulation, especially when compared to the algogenic stimulation group.

It has been indicated that administration of 5-HT potentiated itch sensation by enhancing serotonergic tone [1]. A key finding of the present work was that α-Me-5-HT stimulation increases the level of GABA in the spinal cord. There exist numerous inhibitory interneurons in the spinal cord to regulate the transmission of itch generation [31] and 25-40% of neurons in the spinal laminae I-III are GABAergic neurons [32].

Serotonin is well-known for its ability to facilitate the transmission of descending reticulospinal tracts and GABAergic interneurons [33]. Dergacheva et al demonstrated that multiple applications of the 5-$HT_2$R agonist α-Me-5-HTcaused a long-lasting inhibition of GABAergic neurotransmission within the brainstem [3]. Akiyama et al investigated the role of GABA in the inhibition of spinal itch-signaling neurons in the chronic dry skin itch mouse model and found that scratch-evoked inhibition was markedly attenuated by GABA antagonists [34], indicating that GABA-mediated inhibition is involved in itch-signaling neurons within the spinal cord.

It has been well established fact that the intra-class correlation coefficient of the spinal glutamine- glutamate (GABA) cycle is important in maintaining GABA homeostasis [35, 36]. GABA is a major inhibitory neurotransmitter, whereas glutamate is a major excitatory neurotransmitter in the spinal cord and CNS. Yan et al have reported that the suppression of glial glutamate transporter activities results in an attenuation of glutamate-glutamine cycle-dependent GABA synthesis [37]. Jiang et al demonstrated that the impairment of glial glutamate transporters leads to a reduction of GABA synthesis through the glutamate-glutamine cycle [38]. The comparison between the α-Me-5-HT treated and control groups indicated that the α-Me-5-HT treated group had significantly higher concentrations of GABA and glutamate. It indicated that pruritic stimulation by α-Me-5-HT may cause an increase in the glutamate-glutamine cycle-dependent GABA synthesis.

Numerous pieces of evidences have demonstrated that there are large amounts of GABA/glycinergic mixed inhibitory neurons in the deep dorsal horn of spinal cord [39, 40]. Akiyama et al found that administration of glycine antagonist into the spinal could nearly abolish the scratch-induced inhibition to itch-evoked response (increased spontaneous firing) [41]. Recently, research has concluded that activation of dorsal horn glycinergic neurons could significantly reduce both chemical itch and acute pain [42]. In the current study, glycine took part both in the process of pain and itch, and the changes of the glycine's concentration were inversed. The increased content of glycine might be based on a protective feedback mechanism to resist the increased firings; on the contrary, the decreased glycine of capsaicin group may just be helpful in the delivery of pain signal.

The concentration of taurine decreased in α-Me-5-HT treated samples compared to the controls. Taurine is a critical inhibitory amino acid associated with neuroprotective actions in neural tissue [43, 44]. Terada et al showed in rat models of neuropathic pain that intrathecal administration of taurine resulted in antinociceptive effect [45]. Tee et al also observed that intrathecal taurine had anti-thermal hyperalgesic properties and reduced bicuculline-induced allodynia [46]. Consistent with these findings, our result indicated that the intradermal microinjection of α-Me-5-HT decreased spinal concentrations of taurine, suggesting an important role of spinal taurine during itch proceeding.

Results also suggest that α-Me-5-HT may cause either increased formation or decreased degradation of NAA. NAA is synthesized by N-acetylaspartyl transferase, an enzyme associated with the mitochondria of neurons [47]. It is generally believed that NAA, a marker of neuro/axonal integrity is located exclusively in neurons and their metabolic processes [25, 48]. In the previous reports, a decrease in NAA concentration indicated neuronal dysfunction [48, 49]. Increased function of NAA in the spinal cord after pruritic stimulation by α-Me-5-HT is one of the hypothesized mechanisms leading to neuronal hyperexcitability.

Furthermore, another important finding was that pruritic stimulation by only histamine increased alanine concentrations compared with the control group (2h-13.7%↑, $p$=0.082 and 10min-16.4%↑, $p$=0.014). It is known that alanine plays a key role in the glucose-alanine cycle (also known as the Cahill cycle) in tissues [50, 51]. Recent studies have reported that intradermally injected beta-alanine elicits itch-associated behavior by stimulating receptors expressed on small diameter fibers innervating the skin in mice [52, 53]. It was noted that beta-alanine, an MrgprD agonist, has been shown to play an important role in the sensation of itching. This data could be discrepant with the change of spinal alanine found in our results; differences are probably a result of the pharmacological effects of alanine or beta-alanine. However, the concentration of alanine in the group of α-Me-5-HT does not change significantly. Therefore, further investigations are necessary to clarify the relationships between the changes of spinal alanine, astrocytic involvement and the itch processes underlying different kinds of drugs stimulation.

In summary, the results of the current study are consistent with the hypothesis that the mechanism behind histamine-induced itching is different from that induced by α-Me-5-HT (histamine-independent puritogen). We speculated that the reason may be the different phenotypes provoked by these two compounds. What α-Me-5-HT causes is a pure itch, while histamine leads to a mixture of itching and pain-related sensations [54, 55]. Furthermore, it was evident from our results that the metabolic pattern of pain (capsaicin group) completely differed from that of α-Me-5-HT group, which may help to understand the mechanism of itch alleviation produced by pain stimulation (e.g. capsaicin) [56]. However, the concrete mechanism or relationship between the changes of the metabolites and the scratch behavior need further investigation.

## Conclusions

Results of the present study indicate that proton magnetic resonance spectroscopy was able to simultaneously detect and quantify the absolute concentrations of multiple metabolites within the spinal cord underlying α-Me-5-HT-evoked pruritus. With the stimulation of three different kinds of drugs, the metabolites pattern in the spinal cord significantly differed, especially for α-Me-5-HT and capsaicin. α-Me-5-HT induces its own changes in metabolic specific pattern in the spinal cord, even when compared to another drug, histamine (similar effects but with different mechanism). Application of this magnetic resonance spectroscopy technique will help further explain the mechanism-based understanding of specific itch behavior and improve our knowledge of

itch pathophysiology and pharmacological therapies which may contribute to itch relief.

## Acknowledgments

All the authors would like to express our gratitude to Prof. Yiming Ding (Wuhan Institute of Physics and Mathematics, Chinese Academy of Science) for discussing the results of statistical analysis, Dr. Hongjian He (Zhejiang University) for drawing Fig. 5 (MATLAB code editing), Hansen Wu (Vanderbilt University, USA) for the proofreading. The project was supported by the Chinese Ministry of Science and Technology (2015AA020508), National Natural Science Foundation of China grants 81271766, and National Natural Science Foundation of Hubei Province 2013CFB121.

## Author Contributions

T. Liu, J. Wang, and H. Xiang designed the research; T. Liu, Z. He, G. Kamal, Z. Li, Z. Liu, H. Liu, and J. Wang conducted the experiment; T. Liu, X. Tian, F. Xu, J. Wang, and H. Xiang analyzed the data; T. Liu, J. Wang, and H. Xiang wrote the manuscript. All authors have reviewed and approved the final manuscript.

**Competing financial interests**: The authors declare no competing financial interests.

## References

[1] Z.Q. Zhao, X.Y. Liu, J. Jeffry, W.K. Karunarathne, J.L. Li, A. Munanairi, X.Y. Zhou, H. Li, Y.G. Sun, L. Wan, Z.Y. Wu, S. Kim, F.Q. Huo, P. Mo, D.M. Barry, C.K. Zhang, J.Y. Kim, N. Gautam, K.J. Renner, Y.Q. Li, Z.F. Chen, Descending control of itch transmission by the serotonergic system via 5-HT1A-facilitated GRP-GRPR signaling, Neuron, 84 (2014) 821-834.

[2] C. Liu, T.T. Liu, Z.G. He, B. Shu, H.B. Xiang, Inhibition of itch-related responses by selectively ablated serotonergic signals at the rostral ventromedial medulla in mice, International journal of clinical and experimental pathology, 7 (2014) 8917-8921.

[3] O. Dergacheva, K.J. Griffioen, X. Wang, H. Kamendi, C. Gorini, D. Mendelowitz, 5-HT(2) receptor subtypes mediate different long-term changes in GABAergic activity to parasympathetic cardiac vagal neurons in the nucleus ambiguus, Neuroscience, 149 (2007) 696-705.

[4] Y.G. Sun, Z.F. Chen, A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord, Nature, 448 (2007) 700-703.

[5] Q. Liu, Z. Tang, L. Surdenikova, S. Kim, K.N. Patel, A. Kim, F. Ru, Y. Guan, H.J. Weng, Y. Geng, B.J. Undem, M. Kollarik, Z.F. Chen, D.J. Anderson, X. Dong, Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus, Cell, 139 (2009) 1353-1365.

[6] S.K. Mishra, M.A. Hoon, The cells and circuitry for itch responses in mice, Science, 340 (2013) 968-971.

[7] Y.G. Sun, Z.Q. Zhao, X.L. Meng, J. Yin, X.Y. Liu, Z.F. Chen, Cellular basis of itch sensation, Science, 325 (2009) 1531-1534.

[8] T.C. Glenn, D. Hirt, G. Mendez, D.L. McArthur, R. Sturtevant, S. Wolahan, F. Fazlollahi, M. Ordon, A. Bilgin-Freiert, B. Ellingson, P. Vespa, D.A. Hovda, N.A. Martin, Metabolomic analysis of cerebral spinal fluid from patients with severe brain injury, Acta neurochirurgica. Supplement, 118 (2013) 115-119.

[9] R. Winkler, MASSyPup--an 'out of the box' solution for the analysis of

mass spectrometry data, Journal of mass spectrometry : JMS, 49 (2014) 37-42.

[10] J. Wang, H. Du, L. Jiang, X. Ma, R.A. de Graaf, K.L. Behar, G.F. Mason, Oxidation of ethanol in the rat brain and effects associated with chronic ethanol exposure, Proceedings of the National Academy of Sciences of the United States of America, 110 (2013) 14444-14449.

[11] E. Widerstrom-Noga, Y. Cruz-Almeida, E.R. Felix, P.M. Pattany, Somatosensory phenotype is associated with thalamic metabolites and pain intensity after spinal cord injury, Pain, 156 (2015) 166-174.

[12] E. Widerstrom-Noga, P.M. Pattany, Y. Cruz-Almeida, E.R. Felix, S. Perez, D.D. Cardenas, A. Martinez-Arizala, Metabolite concentrations in the anterior cingulate cortex predict high neuropathic pain impact after spinal cord injury, Pain, 154 (2013) 204-212.

[13] Y. Liu, O. Abdel Samad, L. Zhang, B. Duan, Q. Tong, C. Lopes, R.R. Ji, B.B. Lowell, Q. Ma, VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch, Neuron, 68 (2010) 543-556.

[14] S.G. Shimada, R.H. LaMotte, Behavioral differentiation between itch and pain in mouse, Pain, 139 (2008) 681-687.

[15] T. Akiyama, A.W. Merrill, K. Zanotto, M.I. Carstens, E. Carstens, Scratching Behavior and Fos Expression in Superficial Dorsal Horn Elicited by Protease-Activated Receptor Agonists and Other Itch Mediators in Mice, J. Pharmacol. Exp. Ther., 329 (2009) 945-951.

[16] T. Liu, T. Berta, Z.-Z. Xu, C.-K. Park, L. Zhang, xFc, Ning, Q. Liu, Y. Liu, Y.-J. Gao, Y.-C. Liu, Q. Ma, X. Dong, R.-R. Ji, TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice, The Journal of Clinical Investigation, 122 (2012) 2195-2207.

[17] M. Liu, X.-a. Mao, C. Ye, H. Huang, J.K. Nicholson, J.C. Lindon, Improved WATERGATE Pulse Sequences for Solvent Suppression in NMR Spectroscopy, J Magn Reson, 132 (1998) 125-129.

[18] Y. Liu, J. Cheng, H. Liu, Y. Deng, J. Wang, F. Xu, NMRSpec: An integrated software package for processing and analyzing one dimensional

nuclear magnetic resonance spectra, Chemometrics Intellig. Lab. Syst., 162 (2017) 142-148.

[19] G.M. Kamal, X. Wang, Y. Bin, J. Wang, P. Sun, X. Zhang, M. Liu, Compositional differences among Chinese soy sauce types studied by 13C NMR spectroscopy coupled with multivariate statistical analysis, Talanta, 158 (2016) 89-99.

[20] G. Kamal, B. Yuan, A. Hussain, J. Wang, B. Jiang, X. Zhang, M. Liu, 13C-NMR-Based Metabolomic Profiling of Typical Asian Soy Sauces, Molecules, 21 (2016) 1168.

[21] R.A. de Graaf, G.M. Chowdhury, K.L. Behar, Quantification of high-resolution (1)H-[(1)(3)C] NMR spectra from rat brain extracts, Anal Chem, 86 (2014) 5032-5038.

[22] Y. Liu, Y.-l. Gao, J. Cheng, J. Wang, F.-q. Xu, A Processing Method for Spectrum Alignment and Peak Extraction for NMR Spectra, Chinese J. Magn. Reson., 2 (2015) 382-392.

[23] R. Shank, M. Aprison, Post mortem changes in the content and specific radioactivity of several amino acids in four areas of the rat brain, Journal of neurobiology, 2 (1971) 145-151.

[24] J. Wang, H. Du, X. Ma, B. Pittman, L. Castracane, T.K. Li, K.L. Behar, G.F. Mason, Metabolic products of [2-(13) C]ethanol in the rat brain after chronic ethanol exposure, J Neurochem, 127 (2013) 353-364.

[25] I.L. Simone, F. Federico, M. Trojano, C. Tortorella, M. Liguori, P. Giannini, E. Picciola, G. Natile, P. Livrea, High resolution proton MR spectroscopy of cerebrospinal fluid in MS patients. Comparison with biochemical changes in demyelinating plaques, Journal of the neurological sciences, 144 (1996) 182-190.

[26] N.W. Lutz, S. Maillet, F. Nicoli, P. Viout, P.J. Cozzone, Further assignment of resonances in 1H NMR spectra of cerebrospinal fluid (CSF), FEBS letters, 425 (1998) 345-351.

[27] Y. Wang, D. Li, F. Bao, S. Ma, C. Guo, C. Jin, M. Zhang, Thalamic

metabolic alterations with cognitive dysfunction in idiopathic trigeminal neuralgia: a multivoxel spectroscopy study, Neuroradiology, 56 (2014) 685-693.

[28] J. Braz, C. Solorzano, X. Wang, A.I. Basbaum, Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control, Neuron, 82 (2014) 522-536.

[29] Z.Q. Zhao, F.Q. Huo, J. Jeffry, L. Hampton, S. Demehri, S. Kim, X.Y. Liu, D.M. Barry, L. Wan, Z.C. Liu, H. Li, A. Turkoz, K. Ma, L.A. Cornelius, R. Kopan, J.F. Battey, Jr., J. Zhong, Z.F. Chen, Chronic itch development in sensory neurons requires BRAF signaling pathways, The Journal of clinical investigation, 123 (2013) 4769-4780.

[30] A.D. Papoiu, L.A. Nattkemper, K.M. Sanders, R.A. Kraft, Y.H. Chan, R.C. Coghill, G. Yosipovitch, Brain's reward circuits mediate itch relief. a functional MRI study of active scratching, PloS one, 8 (2013) e82389.

[31] T.C. Sardella, E. Polgar, F. Garzillo, T. Furuta, T. Kaneko, M. Watanabe, A.J. Todd, Dynorphin is expressed primarily by GABAergic neurons that contain galanin in the rat dorsal horn, Molecular pain, 7 (2011) 76.

[32] E. Polgar, T.C. Sardella, M. Watanabe, A.J. Todd, Quantitative study of NPY-expressing GABAergic neurons and axons in rat spinal dorsal horn, The Journal of comparative neurology, 519 (2011) 1007-1023.

[33] I. Hammar, K. Stecina, E. Jankowska, Differential modulation by monoamine membrane receptor agonists of reticulospinal input to lamina VIII feline spinal commissural interneurons, The European journal of neuroscience, 26 (2007) 1205-1212.

[34] T. Akiyama, M. Iodi Carstens, E. Carstens, Transmitters and pathways mediating inhibition of spinal itch-signaling neurons by scratching and other counterstimuli, PloS one, 6 (2011) e22665.

[35] P.I. Ortinski, J. Dong, A. Mungenast, C. Yue, H. Takano, D.J. Watson, P.G. Haydon, D.A. Coulter, Selective induction of astrocytic gliosis generates deficits in neuronal inhibition, Nature neuroscience, 13 (2010) 584-591.

[36] L. Struzynska, G. Sulkowski, Relationships between glutamine, glutamate, and GABA in nerve endings under Pb-toxicity conditions, Journal of inorganic biochemistry, 98 (2004) 951-958.

[37] X. Yan, E. Jiang, H.R. Weng, Activation of toll like receptor 4 attenuates GABA synthesis and postsynaptic GABA receptor activities in the spinal dorsal horn via releasing interleukin-1 beta, Journal of neuroinflammation, 12 (2015) 222.

[38] E. Jiang, X. Yan, H.R. Weng, Glial glutamate transporter and glutamine synthetase regulate GABAergic synaptic strength in the spinal dorsal horn, J Neurochem, 121 (2012) 526-536.

[39] A.J. Todd, R.C. Spike, The Localization of Classical Transmitters and Neuropeptides within Neurons in Laminae-I-Iii of the Mammalian Spinal Dorsal Horn, Progress in neurobiology, 41 (1993) 609-645.

[40] H.U. Zeilhofer, H. Wildner, G.E. Yevenes, Fast Synaptic Inhibition in Spinal Sensory Processing and Pain Control, Physiological reviews, 92 (2012) 193-235.

[41] T. Akiyama, M.I. Carstens, E. Carstens, Transmitters and Pathways Mediating Inhibition of Spinal Itch-Signaling Neurons by Scratching and Other Counterstimuli, PloS one, 6 (2011).

[42] E. Foster, H. Wildner, L. Tudeau, S. Haueter, W.T. Ralvenius, M. Jegen, H. Johannssen, L. Hosli, K. Haenraets, A. Ghanem, K.K. Conzelmann, M. Bosl, H.U. Zeilhofer, Targeted Ablation, Silencing, and Activation Establish Glycinergic Dorsal Horn Neurons as Key Components of a Spinal Gate for Pain and Itch, Neuron, 85 (2015) 1289-1304.

[43] J. Palecek, V. Paleckova, W.D. Willis, The effect of phorbol esters on spinal cord amino acid concentrations and responsiveness of rats to mechanical and thermal stimuli, Pain, 80 (1999) 597-605.

[44] P. Saransaari, S.S. Oja, Taurine and neural cell damage, Amino Acids, 19 (2000) 509-526.

[45] T. Terada, K. Hara, Y. Haranishi, T. Sata, Antinociceptive effect of

intrathecal administration of taurine in rat models of neuropathic pain, Can. J. Anaesth., 58 (2011) 630-637.

[46] I.O. Lee, E.S. Lim, Intracisternal or intrathecal glycine, taurine, or muscimol inhibit bicuculline-induced allodynia and thermal hyperalgesia in mice, Acta Pharmacol. Sin., 31 (2010) 907-914.

[47] J.H. Neale, T. Bzdega, B. Wroblewska, N-Acetylaspartylglutamate: the most abundant peptide neurotransmitter in the mammalian central nervous system, J Neurochem, 75 (2000) 443-452.

[48] F.B. Goldstein, Amidohydrolases of brain; enzymatic hydrolysis of N-acetyl-L-aspartate and other N-acyl-L-amino acids, J Neurochem, 26 (1976) 45-49.

[49] V. Govindaraju, K. Young, A.A. Maudsley, Proton NMR chemical shifts and coupling constants for brain metabolites, NMR in biomedicine, 13 (2000) 129-153.

[50] S. Zhang, G.A. Nagana Gowda, V. Asiago, N. Shanaiah, C. Barbas, D. Raftery, Correlative and quantitative 1H NMR-based metabolomics reveals specific metabolic pathway disturbances in diabetic rats, Analytical biochemistry, 383 (2008) 76-84.

[51] S.M. Houten, H. Herrema, H. Te Brinke, S. Denis, J.P. Ruiter, T.H. van Dijk, C.A. Argmann, R. Ottenhoff, M. Muller, A.K. Groen, F. Kuipers, D.J. Reijngoud, R.J. Wanders, Impaired amino acid metabolism contributes to fasting-induced hypoglycemia in fatty acid oxidation defects, Human molecular genetics, 22 (2013) 5249-5261.

[52] M. Wooten, H.J. Weng, T.V. Hartke, J. Borzan, A.H. Klein, B. Turnquist, X. Dong, R.A. Meyer, M. Ringkamp, Three functionally distinct classes of C-fibre nociceptors in primates, Nature communications, 5 (2014) 4122.

[53] M.V. Valtcheva, S. Davidson, C. Zhao, M. Leitges, R.W.t. Gereau, Protein kinase Cdelta mediates histamine-evoked itch and responses in pruriceptors, Molecular pain, 11 (2015) 1.

[54] E.M. Hartmann, H.O. Handwerker, C. Forster, Gender Differences in Itch

and Pain-related Sensations Provoked by Histamine, Cowhage and Capsaicin, Acta Derm. Venereol., 95 (2015) 25-30.

[55] S.E. Ross, Pain and itch: insights into the neural circuits of aversive somatosensation in health and disease, Curr. Opin. Neurobiol., 21 (2011) 880-887.

[56] T. Patel, G. Yosipovitch, Therapy of pruritus, Expert Opin. Pharmacother., 11 (2010) 1673-1682.

**Figures** :

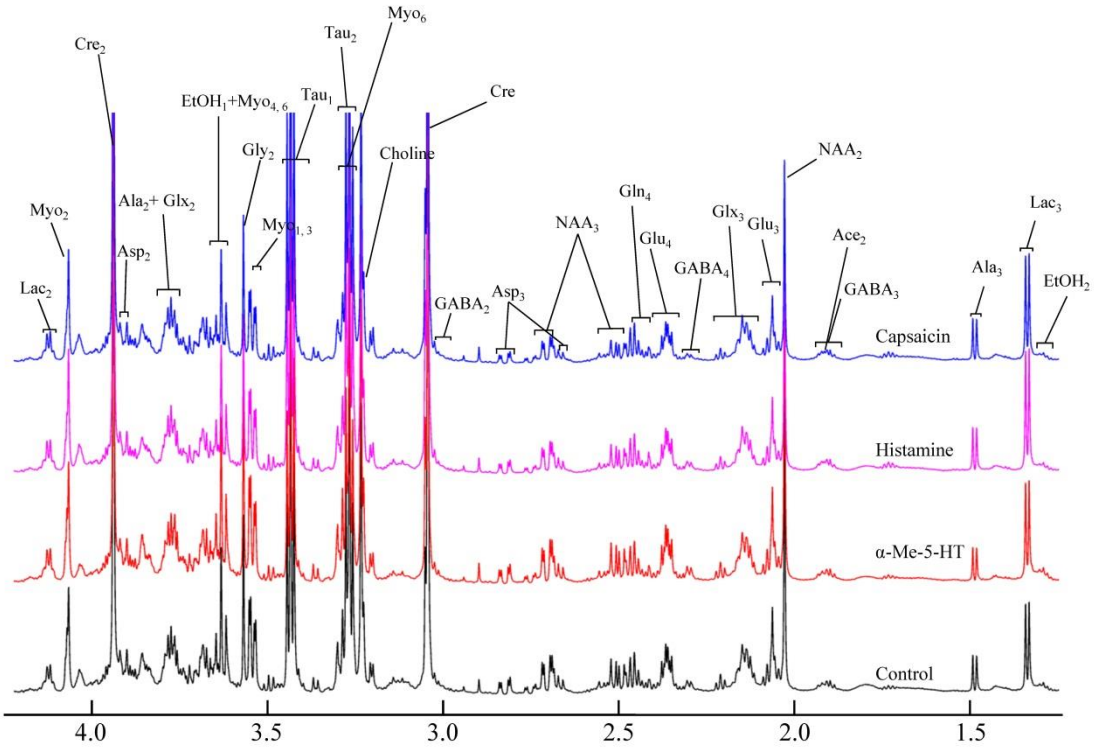



**Figure 1**: The average normalized $^{1}$H-NMR spectra of the spinal cord extracts in the control and treated groups (α-Me-5-HT, histamine and capsaicin). *Note: Lowercase, position of the hydrogen signal which connected with the carbon position; Lac, lactate; Myo: myo-institol; Cre, creatine; Ala, alanine; Glx, glutamine + glutamate; Gly, glycine; Asp, aspartic acid; EtoH, ethanol; Tau, taurine; GABA, gamma amino acid butyric acid; NAA, N-aceytl aspartate; Ace, acetate.*

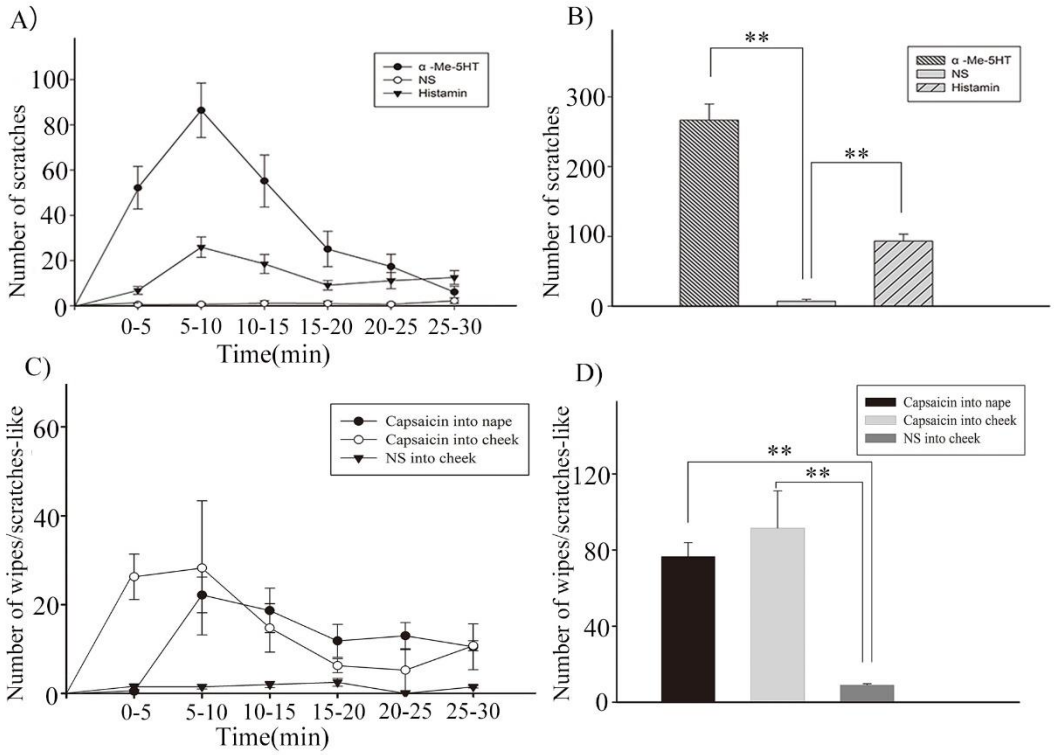



**Fig. 2**: The scratch/wipe behavior of mice in the control, pruritus and pruritogens treated groups. (A) Time course of scratching after injection of α-Me-5-HT (n=10), histamine(n=9) and NS (n=9) in mice. (B) The total number of scratches during a period of 30 min after intradermal injections. (C) Time course of scratches/wipes after injection of saline (cheek, n=4) and capsaicin (cheek, n=4 and nape of neck, n=6)) in mice. (D) The total number of scratches/wipes during a period of 30 min after intradermal injections. Data is represented by means ± SEM. **: $p<0.01$, ANOVA test, followed by LSD post-hoc tests.

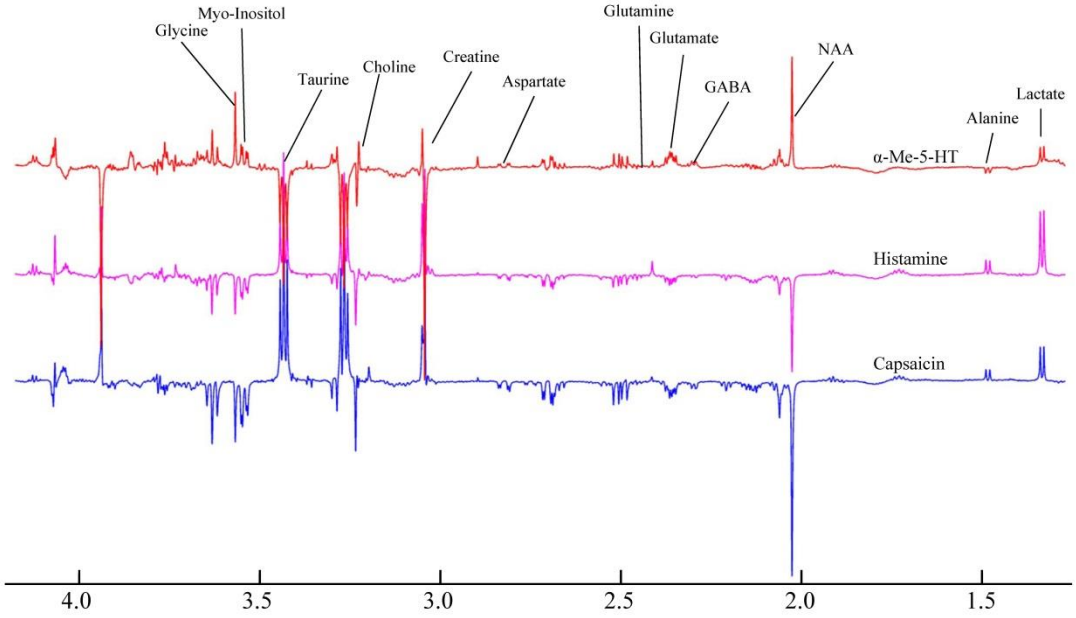



**Fig. 3.** The differences in $^{1}$H-NMR spectra of the spinal cord extracts in the treated groups (α-Me-5-HT, histamine and capsaicin) compared to the control group.

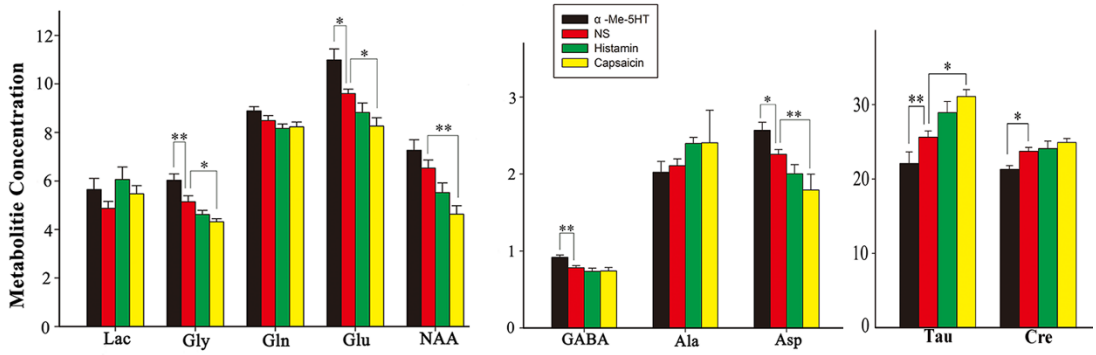
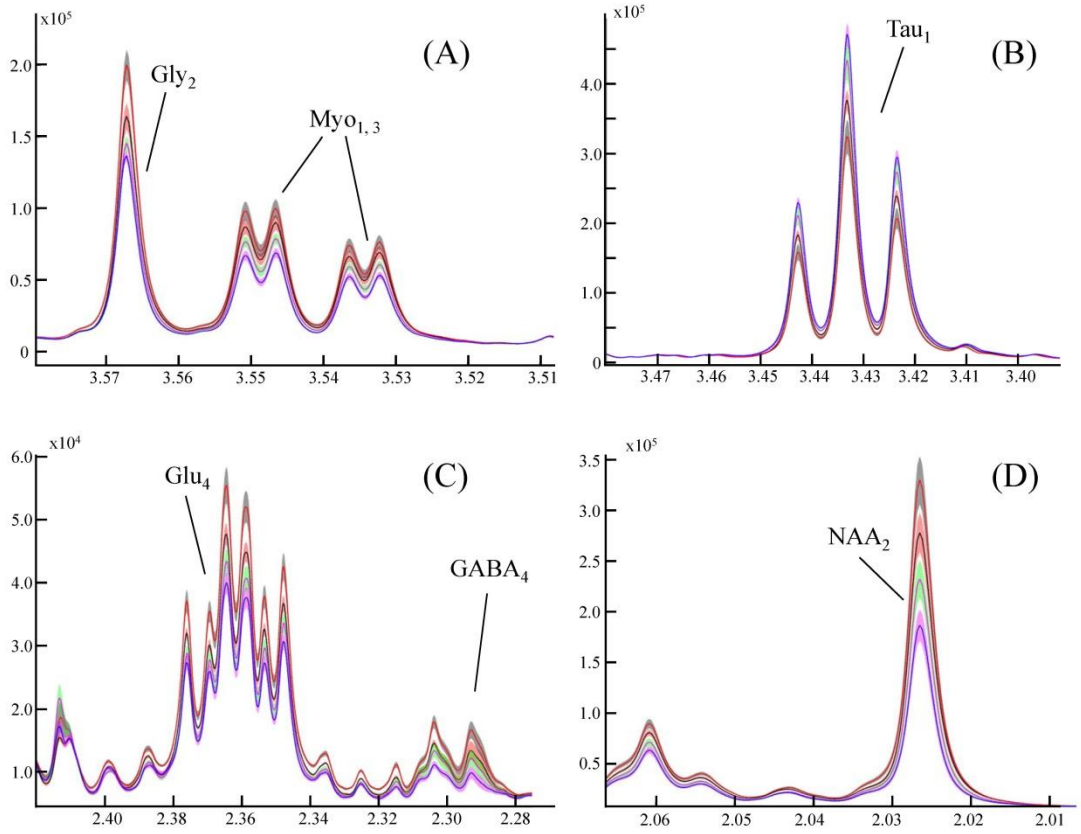



**Fig. 4**: The concentrations of selected metabolites in the spinal cords in the control (NS), and drug treated groups (α-Me-5-HT, histamine and capsaicin). Statistical significance of differences among groups were assessed by one-way ANOVA followed by LSD post-hoc multiple comparison test (*$p$<0.05, **$p$<0.01).




Note: black line: control group; red line: α-Me-5-HT treated; pink line: histamine treated; blue line: capsaicin treated

**Fig. 5**: The difference average normalized spectra of selected metabolites in the control and drug treated groups (α-Me-5-HT, histamine and capsaicin) (means ± SEM). *Note: Black line with red surround: control; Red line with gray surround: α-Me-5-HT-treated group; pink line with green surround: histamine-treated group; blue line with pink surround: capsaicin-treated group.*

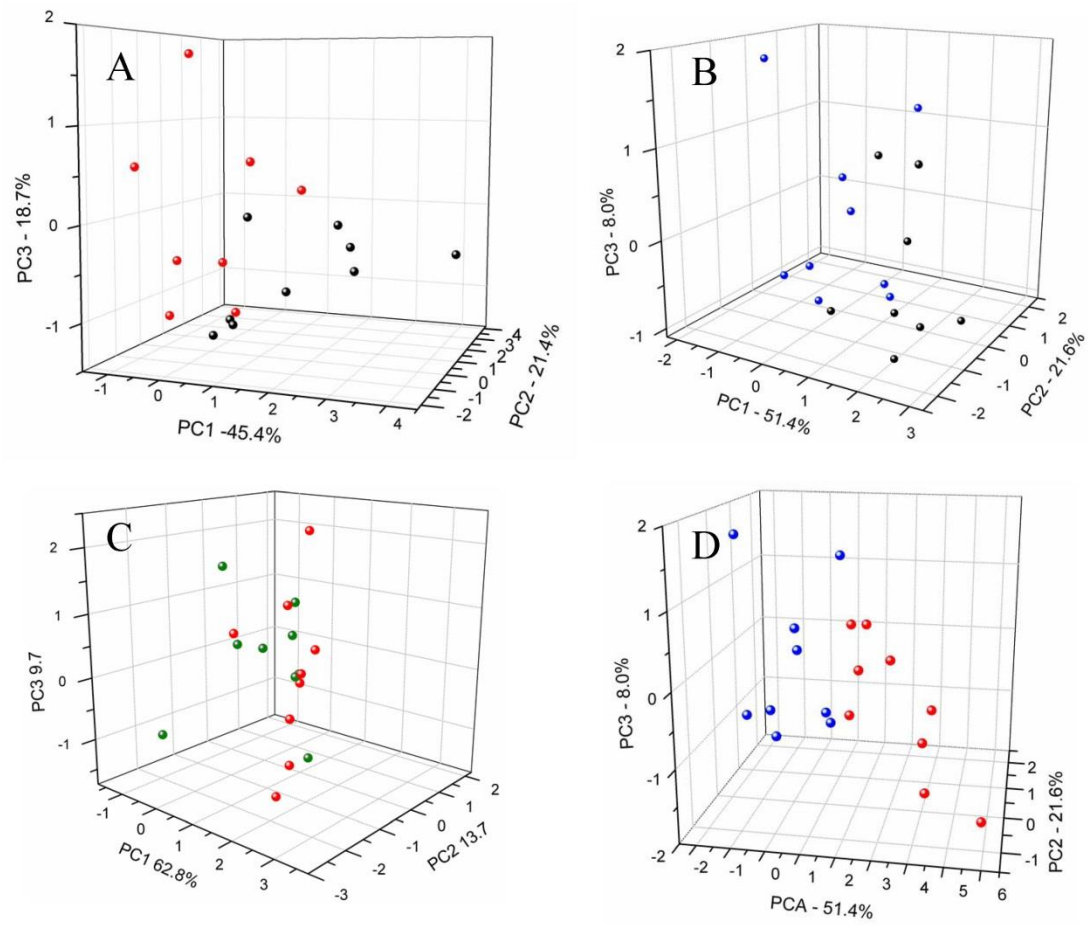
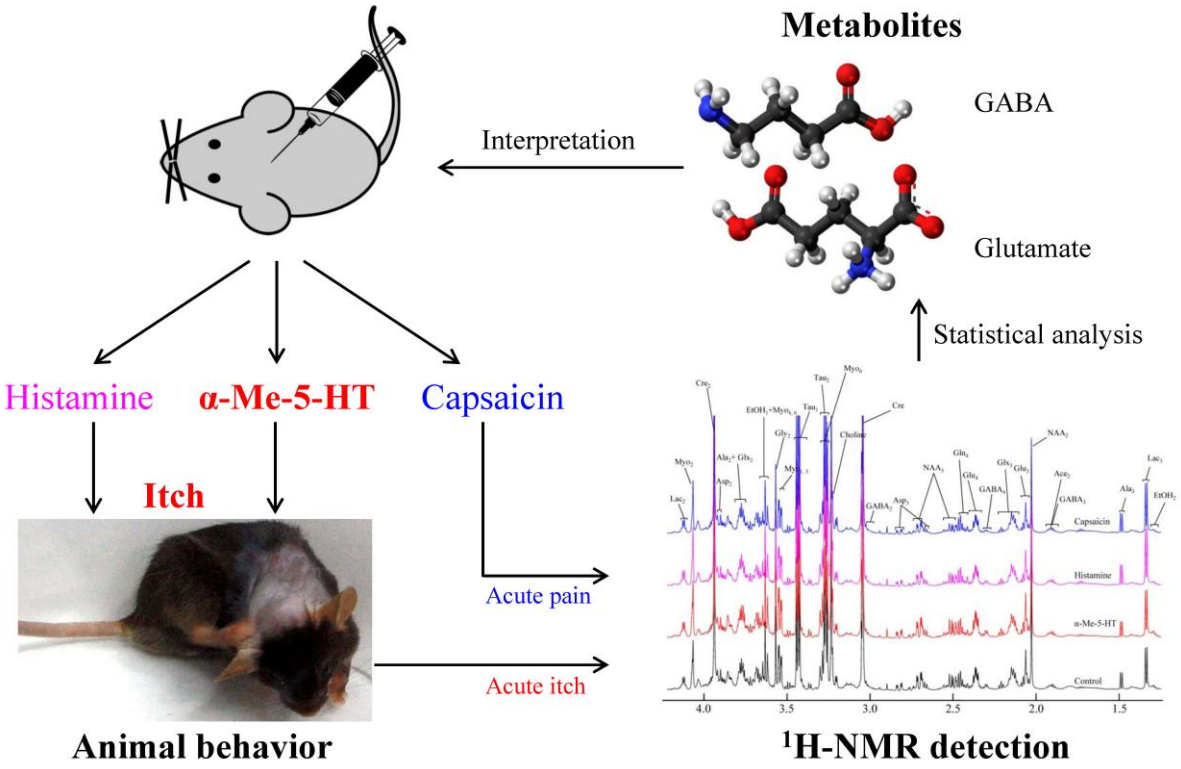



**Figure 6**: Principal component analysis of the spinal cord extracts in the control and α-Me-5-HT, histamine or capsaicin treated groups (Black: control; Red: α-Me-5-HT-treated; Blue: capsaicin-treated; Green: histamine-treated).

Table 1: The correlation coefficients of the different spinal metabolites

| | Lac | NAA | Glu | GABA | Gly | Cre | Myo | Tau | Cho | Asp | Gln | Ala |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lac | 1.000 | 0.174 | 0.241 | 0.450 | 0.065 | -0.320 | 0.042 | -0.341 | -0.053 | 0.201 | 0.185 | 0.027 |
| NAA | 0.174 | 1.000 | **0.959** | 0.568 | **0.823** | -0.590 | **0.915** | **-0.883** | 0.138 | **0.923** | 0.384 | -0.638 |
| Glu | 0.241 | **0.959** | 1.000 | 0.602 | 0.756 | -0.555 | **0.859** | **-0.885** | 0.146 | **0.926** | 0.367 | -0.550 |
| GABA | 0.450 | 0.568 | 0.602 | 1.000 | 0.394 | -0.720 | 0.432 | -0.746 | -0.212 | 0.708 | 0.701 | -0.336 |
| Gly | 0.065 | **0.823** | 0.756 | 0.394 | 1.000 | -0.604 | **0.867** | -0.770 | 0.050 | 0.761 | 0.222 | -0.496 |
| Cre | -0.320 | -0.590 | -0.555 | -0.720 | -0.604 | 1.000 | -0.512 | 0.743 | 0.090 | -0.610 | -0.390 | 0.243 |
| Myo | 0.042 | **0.915** | **0.859** | 0.432 | **0.867** | -0.512 | 1.000 | **-0.830** | 0.061 | **0.850** | 0.261 | -0.687 |
| Tau | -0.341 | **-0.883** | **-0.885** | -0.746 | -0.770 | 0.743 | **-0.830** | 1.000 | 0.008 | **-0.904** | -0.382 | 0.515 |
| Cho | -0.053 | 0.138 | 0.146 | -0.212 | 0.050 | 0.090 | 0.061 | 0.008 | 1.000 | 0.034 | -0.309 | -0.027 |
| Asp | 0.201 | **0.923** | **0.926** | 0.708 | 0.761 | -0.610 | **0.850** | **-0.904** | 0.034 | 1.000 | 0.519 | -0.571 |
| Gln | 0.185 | 0.384 | 0.367 | 0.701 | 0.222 | -0.390 | 0.261 | -0.382 | -0.309 | 0.519 | 1.000 | -0.264 |
| Ala | 0.027 | -0.638 | -0.550 | -0.336 | -0.496 | 0.243 | -0.687 | 0.515 | -0.027 | -0.571 | -0.264 | 1.000 |

Note: Lac: lactate; NAA: N-acetylaspartate; Glu: glutamate; GABA: γ-aminobutyric acid; Gly: glycine; Cre: creatine; Myo: myo-institol; Tau: taurine; Cho: choline; Asp: aspartate; Gln: glutamine; Ala: alaine




**Graphical abstract**

# Highlights

1. Specific pattern of metabolites under α-Me-5-HT evoked pruritus was investigated;
2. $^{1}$H NMR method was utilized to study the change of metabolites in the spinal cord;
3. Normal microwave method was employed to denaturation of the enzymes in the tissue;
4. Metabolic disorders is along with acute pruritic or algogenic stimulation;