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**Differences in neurotransmitter systems of ventrolateral periaqueductal gray  
between micturition reflex and nociceptive regulation:  
an *in vivo* microdialysis study**

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## ABSTRACT

**Objectives:** The midbrain periaqueductal gray (PAG) is a crucial site responsible for the regulation of not only nociception but also the micturition reflex. The physiological functions of the PAG are considered to be under the control of a complex system modulated by several neurotransmitters. However, the system's detailed mechanism has not been clarified.

**Methods:** The present study aimed at elucidating the possible involvement of glutamate and serotonin (5-hydroxytryptamine, 5-HT) neurons in the ventrolateral PAG during noxious stimulation evoked by acetic acid in an animal model of cystitis, focusing on its regulation of the micturition reflex. Changes in glutamate and 5-HT in the PAG during the micturition reflex and acetic acid-induced cystitis were determined using *in vivo* microdialysis combined with cystometry in rats.

**Results:** Extracellular glutamate levels slightly but significantly increased during the micturition reflex induced by saline infusion into the bladder. Intravesical infusion of acetic acid facilitated the micturition reflex characterized by increases in voiding pressure and decreases in the intercontraction interval. Glutamate levels were markedly increased by acetic acid, and this enhancement was sustained for at least three hours. 5-HT levels, which were not altered during the micturition reflex, were increased after intravesical infusion of acetic acid.

**Conclusion:** The results suggest that PAG glutamate and 5-HT neurons differentially participate in the modulation of both nociception and the micturition reflex. Furthermore, PAG 5-HT levels appear to reflect the nociceptive stimuli.

**Keywords:**

Periaqueductal gray; Micturition reflex; Cystitis; Glutamate; Serotonin

**Abbreviations:**

PAG – periaqueductal gray

PMC – pontine micturition center

5-HT – serotonin

## Introduction

The midbrain periaqueductal gray (PAG) is an important processing structure responsible for emotional expression <sup>1</sup>, autonomic responses (respiratory regulation, body temperature control, etc.) <sup>2</sup>, fear <sup>3</sup>, and nociceptive regulation <sup>4</sup>. These physiological functions are regulated by a complex control system modulated by several neurotransmitters. In addition, human studies using functional brain imaging have indicated activation of the PAG during bladder filling <sup>5</sup>. The PAG is also a crucial site for regulating the micturition reflex <sup>6</sup>: based on bladder-filling information, the PAG switches from urine storage to urine voiding. The PAG was thought to activate many descending neurons to the pontine micturition center (PMC), amygdala, thalamus, and hypothalamus <sup>7</sup>, and generate sensations and control the lower urinary tract. Animal studies have shown that the micturition reflex is facilitated by electrical and chemical stimulation of the PAG <sup>8</sup>, and suppressed by chemical stimulation of the PAG <sup>9</sup>. Indeed, the neural mechanism of the PAG has not yet been clarified. Presently, the PAG is divided into four main longitudinal cell-rich subdivisions: the dorsomedial, dorsolateral, lateral, and ventrolateral parts <sup>10</sup>. Taniguchi et al. <sup>8</sup> reported that micturition-inducing sites in the PAG were located in the ventrolateral portion of the caudal PAG. Bladder irritation, i.e., hyperactivity of the bladder detrusor muscle, is well known as being associated with an overactive bladder, interstitial cystitis, and urinary incontinence. Animal studies have shown that chemical irritation induced by intravesical infusion of acetic acid facilitated the micturition reflex and caused severe mucosal degeneration and infiltration of inflammatory cells into the submucosa of the bladder <sup>11</sup>. This noxious stimulation has been widely used as an animal model of cystitis <sup>12</sup>. Mitsui et al. <sup>13</sup> reported that intravesical infusion of acetic acid produced marked increases in c-fos

protein expression in the PAG, as well as in the spinal cord in rats. These findings suggest that the PAG neurons involved in urinary tract functions are induced by noxious stimulation of the bladder. However, the neural mechanism has not been clarified. Pharmacological studies have indicated that glutamate neurons play a key role in the control of urinary tract functions<sup>14</sup>. Serotonergic neurons are important not only in pain modulation, but also in the control of lower urinary tract functions<sup>15</sup>. These findings led us to speculate that glutamate and/or serotonin (5-hydroxytryptamine, 5-HT) neurons in the PAG are responsible for the noxious stimuli-induced micturition associated with bladder inflammation. The present study investigated the possible involvement of glutamatergic and serotonergic neurons in the PAG with normal micturition and noxious stimuli-induced reflex using *in vivo* microdialysis combined with cystometry in freely moving rats. We first investigated the dynamic changes in glutamate and 5-HT levels in the PAG during the micturition reflex induced by saline infusion into the bladder, and then examined the effects of chemical bladder irritation evoked by acetic acid.

## **Methods**

### ***Animals***

Male Wistar rats (10-13 weeks of age; Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were housed in a room with a 12 h light/dark cycle (lights on from 7:00 to 19:00) at  $21 \pm 1^\circ\text{C}$  and were given free access to food and water. All handling of animals was performed in accordance with the Hokkaido University Guidelines for the Care and Use of Laboratory Animals.

### ***Surgical Procedure***

The rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and fixed in a stereotaxic frame, so that bregma and lambda were in the same horizontal plane. After exposure of the brain, a guide cannula was implanted into the ventrolateral PAG (0.8 mm lateral, 7.8 mm posterior to bregma, 4.8 mm ventral to dura) according to the atlas of Paxinos and Watson<sup>16</sup>, and the bladder was exposed through a midline abdominal incision. A polyethylene catheter (PE-50, Clay-Adams, Parsippany, NJ, USA) was inserted into the bladder and sutured bladder wall. The other end of the polyethylene catheter was tunneled subcutaneously and exteriorized through the skin at the animal's back<sup>14</sup>. Each rat was housed singly in a standard plastic cage and allowed to recover from surgery for 4 days. The time schedule of the protocol was determined based on preliminary results and previous reports<sup>17 18</sup>.

### ***Experimental Protocol***

#### ***Microdialysis study***

Microdialysis experiments were conducted in combination with cystometry in freely

moving rats. A dialysis probe (active membrane: 2.0 mm, tip of dialysis probe was projected from the guide cannula) was inserted through the guide cannula and perfused continuously at a flow rate of 2  $\mu\text{L}/\text{min}$  with artificial cerebrospinal fluid (aCSF) (pH 7.4, KCl 2.7, NaCl 140,  $\text{CaCl}_2$  1.2,  $\text{MgCl}_2$  1.0,  $\text{NaH}_2\text{PO}_4$  0.3,  $\text{Na}_2\text{HPO}_4$  1.7 mM) for 60 to 90 min to obtain a stable baseline prior to sampling. Successive samples were collected at 30-min intervals and divided for determination of glutamate and 5-HT. Extracellular levels of glutamate were analyzed by high-performance liquid chromatography (HPLC)-fluorometric detection to form fluorescent derivatives reacted by o-phthaldialdehyde containing mercaptoethanol, as previously described<sup>17</sup>. The mobile phase consisted of 0.1 mM EDTA-2Na/0.1 M-phosphate buffer adjusted to pH 6.0 and methanol in a v/v proportion of 7:3. Extracellular 5-HT levels were determined using HPLC with electrochemical detection (ECD-300, Eicom Co. Ltd., Kyoto, Japan). A working electrode was maintained at 450 mV. The mobile phase consisted of 2.1 mM sodium 1-decansulfonate, 0.1 mM EDTA-2Na/0.1 M-phosphate buffer adjusted to pH 6.0, and 1% (v/v) methanol. Using an HPLC-electrochemical detection system, the total amount of all peaks is called a chromatogram. Each individual peak provides qualitative and quantitative information of glutamate and 5-HT.

At the end of the experiment, the rats were deeply anesthetized with pentobarbital and perfused with saline followed by a mixture containing 0.9% saline and 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Blocks of brain tissue containing the PAG were removed, and dialysis probe placement was confirmed histologically as previously described<sup>18</sup>. Only data from rats whose probe placements were in the ventrolateral region were used for analysis.

### *Cystometry recording*

Throughout the microdialysis experiment, the bladder catheter was connected to a TE-332S syringe pump (Terumo, Tokyo, Japan) and to a TSD104A pressure transducer (Biopac Systems, Santa Barbara, CA, USA) for bladder pressure monitoring.

Physiological saline or 0.1 % acetic acid was infused at a rate of 0.2 ml/min for 30 min at room temperature. The micturition reflex was evaluated by cystometric parameters, such as intercontraction interval and maximal voiding pressure (pressure triggering the micturition reflex), using AcqKnowledge 3.7.1 software (Biopac Systems, Santa Barbara, CA, USA).

### *Statistics*

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Analysis of cystometric parameters was determined by Student's t-test for unpaired data. Analysis of the time course was performed by repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc test. Where applicable, Student's unpaired t-test was used. Results with  $P < 0.05$  were considered significant.

## Results

### *Cystometry*

When saline was continuously infused into the bladder, the micturition reflex occurred.

Intravesical infusion of acetic acid markedly increases the maximal voiding pressure and decreases the intercontraction interval were also observed (Fig. 1A). Statistical analysis showed significant decreases in the intercontraction interval in the acetic acid-induced cystitis model, when compared with those in the saline-infused controls ( $p < 0.05$ ) (Fig. 1B). These results indicate that the micturition reflex was facilitated by the intravesical infusion of acetic acid under the current conditions.

### *Changes in glutamate and serotonin in the ventrolateral PAG during the micturition reflex and acetic acid-induced cystitis*

Changes in the ventrolateral PAG glutamate and 5-HT levels determined in combination with cystometry are shown in Fig. 2. Glutamate levels increased slightly but significantly during the micturition reflex induced by saline infusion at 30 min in the freely moving rats ( $p < 0.05$  compared with non-treated controls and with baseline), whereas the 5-HT levels did not change. When acetic acid was infused into the bladder, marked increases in the glutamate levels were observed in both the anesthetized and freely moving rats, and significant enhancement was sustained up to 180 min ( $p < 0.05$  compared with saline infusion and with baseline). The 5-HT levels also increased significantly during the infusion of acetic acid, but gradually subsided one hour after the infusion ( $p < 0.05$  compared with saline infusion and with baseline) (Fig. 2). Consistent with previous reports, the basal serotonin level continued to decrease over time<sup>17, 18</sup>.

## Discussion

To the best of our knowledge, this is the first report showing changes in extracellular levels of glutamate and serotonin in the ventrolateral PAG during the micturition reflex and acetic acid-induced cystitis in rats. Initially, we performed this protocol under the anesthetized condition. Not only cystometric parameters but also changes in neurotransmitters were suppressed (data not shown). The anesthetized condition could influence the neural activity of the PAG. In this paper, we performed the protocol on the freely moving rats.

Very few reports can be found regarding micturition-related neuronal activity. Liu et al. reported that micturition-related neuronal firings in both the PAG storage and micturition cycles using cats<sup>19</sup>. Although, this report describes evidence of single neuronal activity in the PAG occurring in close association with the micturition reflex, the authors use the response to isovolumetric spontaneous micturition reflexes in supracollicular decerebrated cats. Furthermore, our group previously reported that bladder contractions and external urethral sphincter activity were controlled by a injections of drug into the PAG<sup>20</sup>. The PAG is a crucial site responsible for regulating many physiological functions, of which both nociception and the micturition reflex are very important for maintaining vital functions. Even with the most advanced functional brain imaging technology, it is difficult to identify and discriminate between excitation and inhibition of brain regions. The information of time-dependent changes of neurotransmitters enables a deeper understanding of this crucial site. We found that extracellular levels of glutamate in the PAG were increased during the micturition reflex induced by saline infusion into the bladder. These changes were only transient, which confirms the conclusions of our previous study<sup>18</sup> and suggests that the glutamatergic

neurons in the PAG have a facilitatory role in regulating the micturition reflex. This is consistent with a report demonstrating that microinjection of glutamate into the PAG elicited the micturition reflex <sup>21</sup>.

In contrast, 5-HT neurons are considered to play an important role as inhibitory neurotransmitters in the micturition reflex pathway <sup>22</sup>. For instance, electrical stimulation of 5-HT-containing neurons in the raphe nuclei inhibited the micturition reflex, presumably via 5-HT release <sup>23</sup>. In the present study, the level of dialysate 5-HT, as well as glutamate, decreased in response to the removal of calcium from the perfusate and increased in response to the addition of potassium (data not shown), indicating that these neurotransmitters originate from nerve endings. Furthermore, in our previous report <sup>18</sup>, glutamate and dopamine levels increased significantly and gamma-aminobutyric acid (GABA) levels decreased significantly in a reproducible manner, in parallel with the micturition reflex. However, in the present study, we did not observe significant changes in 5-HT levels during the micturition reflex. Therefore, 5-HT neurons, at least in the PAG, are unlikely to play a predominant role in regulating the micturition reflex under our specific conditions.

Conversely, glutamate and 5-HT in the PAG were markedly increased by the intravesical infusion of acetic acid, indicating that both neurotransmitters are involved in the supraspinal nociceptive mechanism. Cystometry showed that the infusion of acetic acid elicits irritative bladder responses characterized by increased maximal voiding pressure and decreased intercontraction interval. Increases in glutamate levels induced by acetic acid are longer lasting than are increases induced by the saline-induced micturition reflex. Thus, the facilitation of glutamate observed during the infusion of acetic acid appears to be attributable to the modulation of the micturition

reflex with noxious stimulation. A plausible explanation for these results, therefore, is that the noxious signal in the bladder evoked by the acetic acid is transmitted to the PAG, and the glutamate can then modulate the organization of the micturition reflex and trigger the initial step of developing irritative bladder responses. Intravesical infusion of acetic acid induces two-way information from the bladder. The first is increased bladder pressure. This information is relayed by A $\delta$  fibers to the sacral cord, where they terminate on Gert's nucleus<sup>24</sup>, which cell group relays this information to the PAG. Secondly, cystitis information induced by the acetic acid is relayed via C-fibers, which terminate on lamina I and V neurons of the sacral cord, which may relay this information to the PAG and activate critical top-down brainstem spinal cord modulatory processes. However, the mechanisms underlying this two-differential modulation remain unknown. Numerous studies have shown that glutamate neurons participate in different types of noxious stimulation. Silva et al.<sup>25</sup> reported that formalin injections in rat hind paw increased PAG glutamate levels. The authors suggested that increases in glutamate are responsible for persistent pain, rather than stress or short-duration noxious stimulation, such as pinching. Although the functional significance of the facilitation of glutamate underlying persistent noxious stimulation has not been clarified, it may be related to pain suppression by PAG activation<sup>26</sup>. Indeed, it may be microinjection of glutamate or a glutamate agonist-induced analgesia<sup>27</sup>. These findings, combined with the present results, suggest that activation of the PAG caused by noxious stimulation in the bladder may be attributable to increased glutamate release as part of the analgesic mechanism.

Interestingly, PAG glutamate and 5-HT appear to be differentially involved. Increases in 5-HT levels induced by acetic acid gradually decreased, but increases in glutamate

release were prolonged. These results suggest that 5-HT neurons might participate in the control of relatively early-stage bladder inflammation. Zhang et al.<sup>28</sup> demonstrated that increases in PAG 5-HT levels correspond to inflammation, and that the increasing time in the PAG appears to be earlier than that in the spinal cord. Cui et al.<sup>29</sup> reported that PAG stimulation caused increases in 5-HT release in the spinal differential 5-HT mechanism in the PAG, and that underlying noxious stimulation of the spinal cord appears likely. Hypothetically, sustained increases in glutamate levels might be related to pain suppression induced by PAG activation, whereas increases in 5-HT might respond to nociceptive stimuli. Moreover, previous researchers reported that stimulation of PAG's group II metabotropic glutamate receptor increases 5-HT release, while stimulation of NMDA glutamate receptors decrease it<sup>30</sup>. The interaction between glutamate and 5-HT might be modulating in the PAG in the micturition reflex and acetic acid-induced cystitis in rats. However, we still do not understand the detailed mechanisms of this interaction (including the presence of interneurons). This is the subject of future investigation.

In conclusion, the present study revealed that both glutamate and 5-HT neurons in the PAG participate in the supraspinal nociceptive response induced by chemical bladder irritation evoked by acetic acid. Glutamate neurons, but not 5-HT neurons, contribute to the regulation of both noxious stimuli-induced micturition and the micturition reflex. To the best of our knowledge this is the first report showing that dynamic changes in endogenous glutamate and 5-HT in the PAG occurred during bladder inflammation in freely moving rats. Understanding the role of these neurotransmitters in the modulation of noxious stimuli-induced micturition accompanied with inflammation might provide

further insight into the pathophysiology of persistent cystitis, including interstitial cystitis.

**Conflict of interest statement**

The authors declare that they have no competing interests.

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## Figure legends

### Figure 1

(A) Representative cystometrography of the micturition reflex in a freely moving rat (awake). Physiological saline or acetic acid was infused.

(B) Changes in cystometric parameters in the saline-infused controls and acetic acid-induced cystitis model in free moving rats. Data are expressed as mean  $\pm$  SEM.

Student's t-test was used for the comparison of saline and acetic acid infusion \*P < 0.05

(n = 6 rats) data values compared with control.

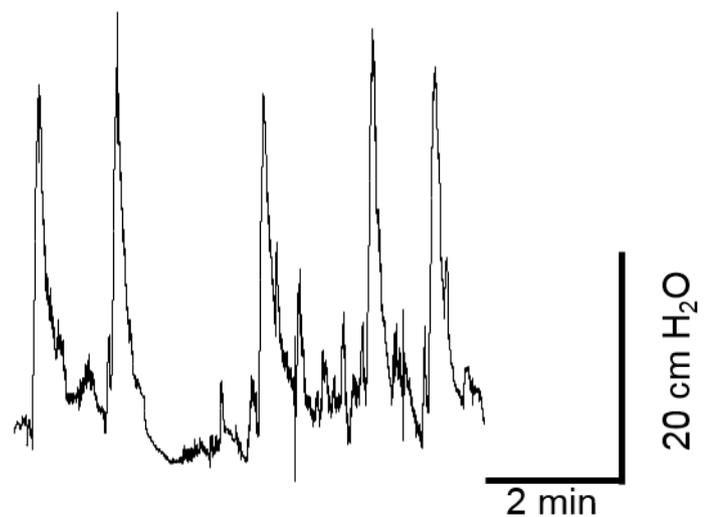
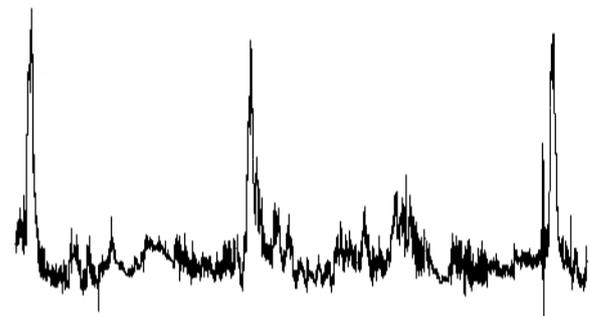
ICI, intercontraction interval; MVP, maximal voiding pressure.

### Figure 2

Time-course response of extracellular levels of glutamate and serotonin in the PAG after acetic acid or saline infusion into the bladder in free moving rats. Data are expressed as mean  $\pm$  SEM. Statistical analysis of the time course was performed by repeated measure analysis of variance (ANOVA) followed by Tukey's post hoc test. # P<0.05 compared with non-treated controls (Control). \* P<0.05 compared with saline infused into the bladder (Saline). †P < 0.05 vs. basal levels before acetic acid or saline infusion.

Fig.1

(A)



(B)

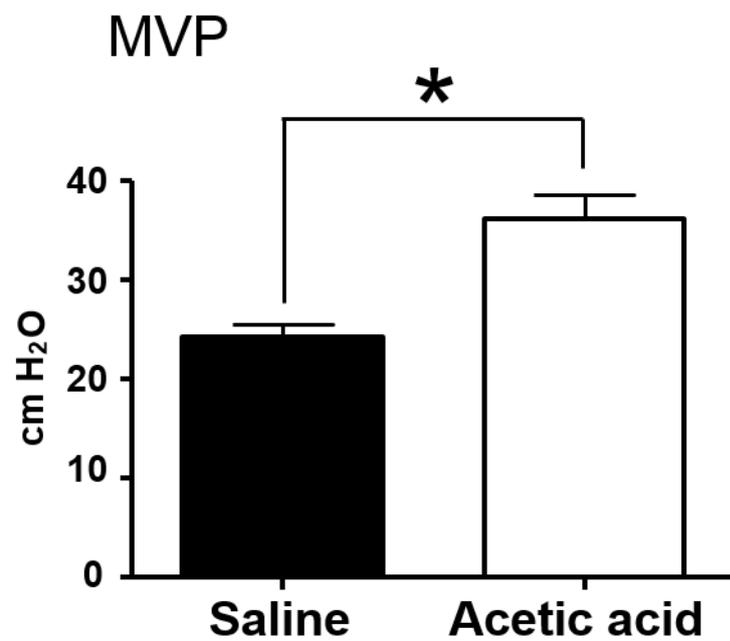
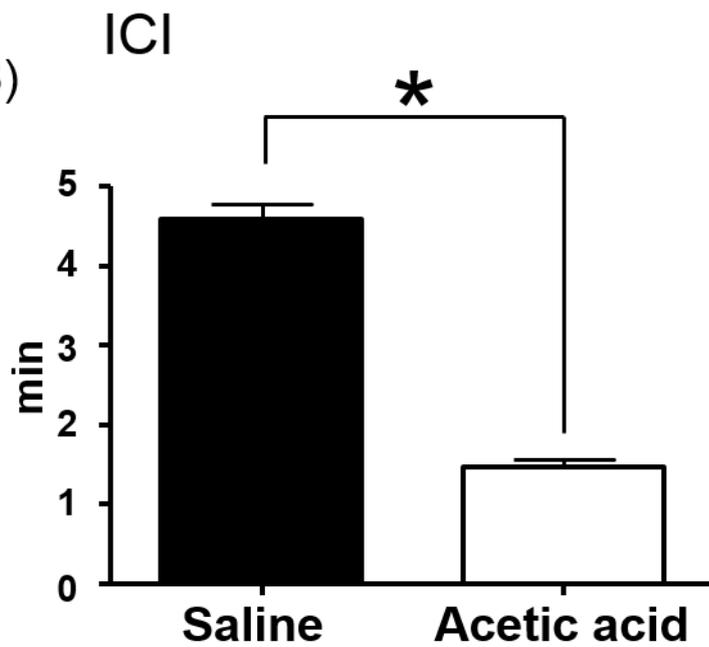
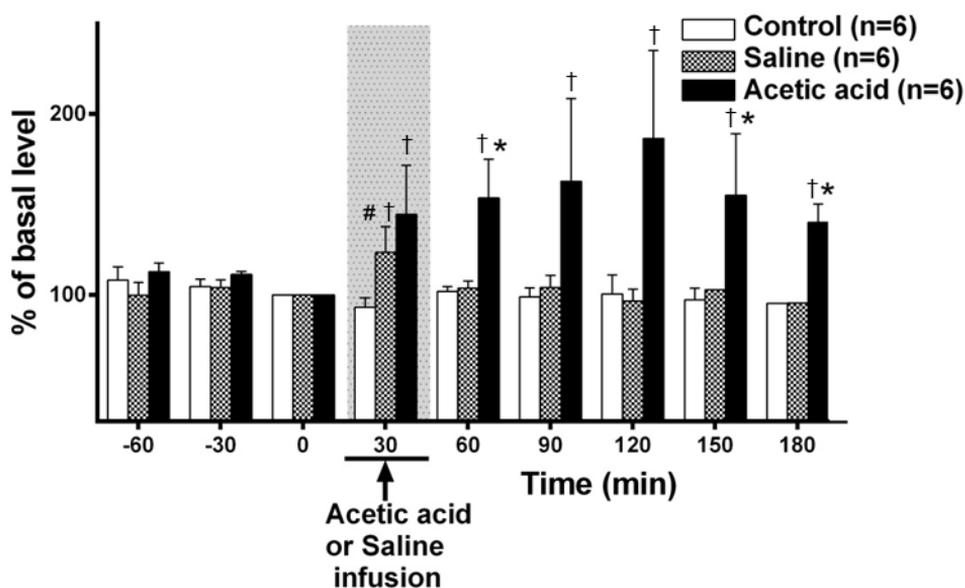


Fig.2

## Glutamate



## Serotonin

