

# GABA<sub>A</sub> receptor overexpression in the lateral hypothalamic area attenuates gastric ischemia-reperfusion injury in rats

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**Abstract.** Excessive activation of the greater splanchnic nerve (GSN) has previously been determined to contribute to the progression of gastric ischemia-reperfusion (GI-R) injury. The present study was designed to estimate the protective effects of GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) overexpression in the lateral hypothalamic area (LHA) against GI-R injury. The GI-R injury model was induced in rats by clamping the celiac artery for 30 min and then reperusing for 1 h. Microinjection of recombinant adenoviral vectors overexpressing GABA<sub>A</sub>R (Ad-GABA<sub>A</sub>R) or control adenoviral vectors (Ad-Con) into the LHA was conducted in GI-R and normal control rats. Significant protective effects were observed on day 2 after Ad-GABA<sub>A</sub>R treatment in the GI-R injury rats. Ad-GABA<sub>A</sub>R treatment reduced plasma norepinephrine levels, plasma angiotensin II levels and peripheral GSN activity, but increased the gastric mucosal blood flow, as compared with Ad-Con treatment. These results indicate that adenoviral vector-induced GABA<sub>A</sub>R overexpression in

the LHA blunts GSN activity and subsequently alleviates the effects of gastric injury in GI-R rats.

## Introduction

Major surgery-evoked ischemia has been demonstrated to induce gastric mucosal injury and gastrointestinal dysmotility (1,2). Previous studies (3-5) have shown that the hypothalamic paraventricular nucleus and the lateral hypothalamic area (LHA) are two specific hypothalamic nuclei that modulate gastric activity and gastric mucosal injury. The microinjection of GABA<sub>A</sub> receptor blocker in the LHA enhances GI-R injury. However, little is known regarding GABA<sub>A</sub> receptor expression and the protective effects of GABA<sub>A</sub>R overexpression in the LHA against gastric ischemia-reperfusion (GI-R) injury in rats. As determined by a previous study (6), cerebellar-hypothalamic circuits regulate the gastric mucosal injury induced by ischemia-reperfusion. However, the detailed GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)-mediated regulative mechanism in the LHA upon GI-R injury is not clear. In the present study, the effects of GABA<sub>A</sub>R overexpression induced by recombinant adenovirus vectors in the LHA following GI-R injury in rats were investigated. The aim of this study was to investigate the potential mechanisms of the GABA<sub>A</sub> receptor in GI-R injury.

## Materials and methods

**Animals.** Adult male Sprague Dawley (SD) rats were obtained from the Animal Resource Centre (Fudan University, Shanghai, China). All experimental procedures used in this study were performed in accordance with the Experimental Animal Care and Use Committee of Fudan University and conformed to the guidelines set out by the National Health and Medical Research Council of China. All rats were housed under controlled conditions (12 h light initiated at 20:00; 22-24°C)

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and provided access to water *ad libitum* for the duration of the study. The animals were fasted for 24 h prior to the experiment and were then allocated to the different groups. Following the experiments, the animals were deeply anesthetized with 10% chloral hydrate (solvent, 0.9% normal saline) and euthanized by cervical dislocation followed by decapitation.

**Viral microinjection.** Rats were anesthetized and placed in a stereotaxic frame (51600; Stoelting, Chicago, IL, USA). The recombinant adenoviral vectors overexpressing GABA<sub>A</sub>R (Ad-GABA<sub>A</sub>R; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or control adenoviral vectors (Ad-Con) were bilaterally microinjected into the LHA (15  $\mu$ l for each side). The stereotaxic coordinates of LHA:LHA:AP 2.8 mm, LR: 1.5 mm, H: 8.3–8.5 mm, which was in accordance with Paxinos & Watson's rat atlas ([http://www.callisto-science.org/NSI/Neuroscience\\_Image\\_Database/Rat\\_Brain\\_Atlas.html](http://www.callisto-science.org/NSI/Neuroscience_Image_Database/Rat_Brain_Atlas.html)).

**GI-R injury model.** GI-R was performed following the microinjection of recombinant adenoviral vectors into the LHA according to previously reported methods (7). In brief, the abdominal cavity was cut open and the celiac artery was carefully isolated from the adjacent tissues. The celiac artery was clamped with a small vascular clip for 30 min and then reperfusion was established by removal of the clip for 1 h.

Control animals underwent an identical surgical procedure with the exception of clamping the celiac artery. At the end of the experiments, the rats were sacrificed as described. The stomachs were rapidly removed and were cut open along the greater curvature, then the gastric mucosa was carefully assessed for ulcers.

**Assessment of gastric mucosal injury index (GMII).** Gastric mucosal injury was measured as previously described (6). The stomach was incised along the greater curvature and washed with phosphate-buffered saline. The GMII was determined by a cumulative-length scale, in which an individual lesion limited by the mucosal epithelium, including the pinpoint erosions, ulcers and hemorrhagic spots, was scored according to length. The scores were calculated using the following criteria: 1, lesions  $\leq 1$  mm; 2, lesions  $> 1$  mm and  $\leq 2$  mm; 3, lesions  $> 2$  mm and  $\leq 3$  mm. For lesions of width  $> 1$  mm, the lesion score was doubled. The sum of the scores indicated the GMII. To prevent researcher bias, the GMII was measured by a researcher who was blind to the treatments.

**Measurement of greater splanchnic nerve (GSN) activity.** The rat was anesthetized with chloral hydrate (400 mg/kg intraperitoneally) and mounted onto a stereotaxic apparatus. The LHA coordinates were as described. Ad-GABA<sub>A</sub>R (15  $\mu$ l for each side) was microinjected via a cannula connected to a microsyringe with a polyethylene tube. The injection lasted 2 min and the injection cannula was left for an additional 10 min to prevent backflow. The central end of the GSN was placed on thin bipolar platinum electrodes. The nerve-electrode junction was fixed and was electrically insulated from the surrounding tissues with a Vinyl Polysioxane Impression Material-auto-mixture (Sigma-Aldrich, St. Louis, MO, USA). The rectified output from the amplifier was monitored using the PowerLab<sup>®</sup> system (ADInstruments, Dunedin, New

Zealand) to record the raw nerve discharge. The basal nerve activity (baseline) was determined by analysis of the efferent GSN activity at the beginning of the experiment and background noise was measured by the nerve activity recorded at the end of the experiment. The nerve activity during the experiment was calculated by subtracting the background noise from the recorded value. The GSN activity response to the Ad-GABA<sub>A</sub>R treatment was expressed as the percentage change from the basal value.

**Gastric mucosal blood flow (GMBF) measurement.** The GMBF was analyzed with a laser-Doppler flowmeter (LDF-2; Nankai University, Tianjin, China). In brief, the rats were anesthetized with chloral hydrate (400 mg/kg intraperitoneally), the abdomen was opened, the stomach was exposed and transected, and the gastric contents were marginally evacuated to the exterior through the 5 mm incision in the stomach. Subsequently, the laser probe was placed 0.5 mm above and perpendicular to the mucosal surface to monitor GMBF, with measurements expressed in mV (value of Doppler signal voltage) on the digital panel of the flowmeter. When the GMBF was stable, four points for measurement were selected (one point for 1 min), and the average value was calculated and expressed as U/mV.

**Western blot analysis of GABA<sub>A</sub>R expression levels.** Sample brain tissues were collected and homogenized in RIPA lysis buffer. The concentration of total protein was detected by bicinchoninic acid assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The proteins (40  $\mu$ g) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). The membranes were probed with primary antibody to GABA<sub>A</sub>R (1:200; Santa Cruz Biotechnology, Inc.) for 2 h. Next, the membranes were washed three times with phosphate-buffered saline and incubated with goat anti-rabbit IgG (1:1,000) secondary antibody for 2 h. Following incubation with enhanced chemiluminescence solution (Pierce Biotechnology, Inc., Rockford, IL, USA) and visualization by exposure to BioMax films (Kodak, Rochester, NY, USA), the membranes were stripped and probed with mouse monoclonal anti- $\beta$ -actin primary antibody (1:300; Santa Cruz Biotechnology, Inc.) and rabbit anti-mouse secondary antibody for 2 h. The results are expressed as the optical density of the experimental band divided by that of the  $\beta$ -actin band of four replicate experiments. The optical density was measured by a gel-pro analyzer (Shanghai Furi Science & Technology Co., Ltd., Shanghai, China).

**Measurement of plasma norepinephrine (NE).** Blood samples were obtained from the carotid artery, through a tube that contained ETDA. The sample was centrifuged and mixed in an antioxidant stabilizer sodium metabisulphite solution (5.2 mM). The plasma NE level was determined by high-performance liquid chromatography (HPLC) using a YWG-C18 column (250 mm x 4.6 mm x 5  $\mu$ m) and electrochemical detection (Waters 2465; Waters Corporation; Milford, MA, USA) as previously reported (8).

**Measurement of plasma angiotensin (Ang) II.** Plasma Ang II levels were analyzed using commercial ELISA kits (R&D

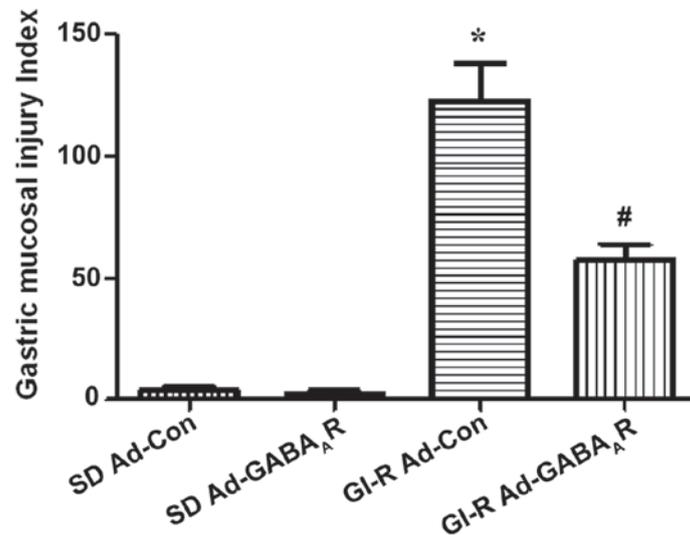


Figure 1. Effects of Ad-GABA<sub>A</sub>R on GI-R injury in rats. \*P<0.05, as compared with the SD Ad-Con group. #P<0.05, as compared with the GI-R Ad-Con group. n=8 in each group. Ad-GABA<sub>A</sub>R, adenoviral vectors overexpressing GABA<sub>A</sub> receptor; GI-R, gastric ischemia-reperfusion; SD, Spague Dawley; Ad-Con, control adenoviral vector.

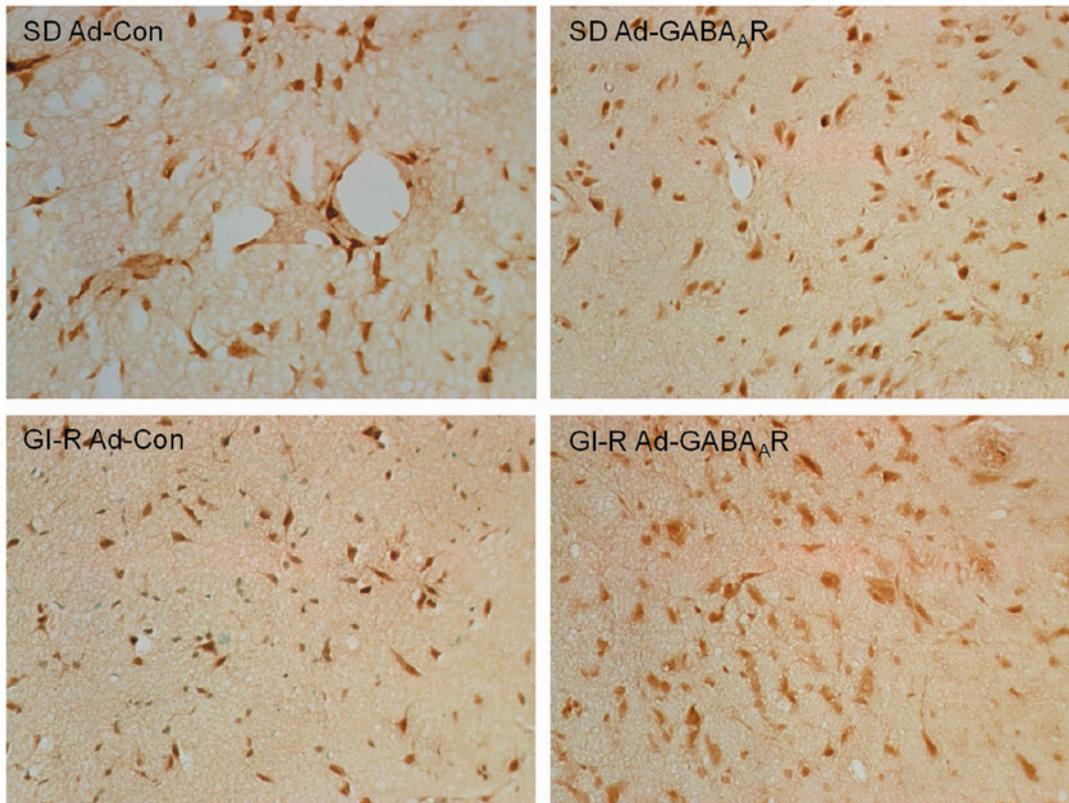


Figure 2. GABA<sub>A</sub>R expression and cellular localization were detected by immunohistochemistry. As compared with the control group, GABA<sub>A</sub>R was upregulated in the Ad-GABA<sub>A</sub>R groups. GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; Ad-GABA<sub>A</sub>R, adenoviral vectors overexpressing GABA<sub>A</sub>R; Ad-Con, control group.

systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, following incubation of 96-well plates with antibody specific for rat Ang II (1:500), the samples and standard diluent buffer were added to the wells, which were subsequently incubated and washed. Horseradish peroxidase-conjugated solution (1:100) was added and then washed out. The reactions were terminated with stop solution and the final solution was read at 450 nm using an ELISA

plate reader (Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China).

**HPLC.** The amino acids in the microdialysis sample were separated by HPLC (LC-6A UQUID Chromatograph; Shimadzu Biotech Corporation, Kyoto, Japan) using a reverse-phase column (C18; Ultrasphere ODS; 4.6 mmx25 cm; 5 μm particles), and quantified by o-phthalaldehyde derivative and

fluorescence detection (RF-10AXL Shimadzu Fluorescence detector; 0.01 relative fluorescence units, 330 nm excitation wave-length and 450 nm emission wave-length). The mobile phase consisted of 0.1 M 63% potassium phosphate (pH 6.00, 6.25), 35% methanol and 2% tetrahydrofuran, and the flow rate was 1 ml/min. The experiments were conducted at 19–23°C (9).

**Histology.** Following the experiments, the rats were euthanized by an overdose injection of urethane followed by thoracotomy. The brain was removed from the skull, fixed in 10% formalin for four days or used to produce 40 mm coronal frozen sections and stained with GABA<sub>A</sub>R antibody for immunohistochemical analysis, as previously reported (6).

**Statistical analysis.** A student's t-test and one-way analysis of variance was used for data analysis, and the data are presented as the means ± standard error of the mean. GraphPad 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used and a  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of Ad-GABA<sub>A</sub>R on GI-R injury.** It was first determined whether the overexpression of GABA<sub>A</sub>R in the LHA exerted a protective effect upon GI-R injury. Microinjection of Ad-GABA<sub>A</sub>R into the LHA was found to attenuate GI-R injury. The GMII that followed Ad-GABA<sub>A</sub>R microinjection into the LHA, was significantly reduced as compared with the GMII subsequent to Ad-Con injection ( $122.7 \pm 15.6$  versus  $57.0 \pm 6.67$ ;  $P < 0.05$ ; Fig. 1).

**Effects of Ad-GABA<sub>A</sub>R microinjection into LHA on GABA<sub>A</sub>R expression.** In order to detect the depressive effects of Ad-GABA<sub>A</sub>R on the expression of GABA<sub>A</sub>R in the LHA, GABA<sub>A</sub>R expression and cellular localisation were detected by immunohistochemistry. The results revealed that GABA<sub>A</sub>R expression was upregulated by microinjection of Ad-GABA<sub>A</sub>R in the normal SD group and the GI-R group (Fig. 2). The recombinant adenoviral vectors encoding GABA<sub>A</sub>R significantly increased the expression levels of GABA<sub>A</sub>R in the LHA at two days after the viral microinjection in GI-R and normal SD rats ( $P < 0.05$ ; Fig. 3).

**Plasma Ang II and NE levels.** Plasma Ang II was significantly increased in the GI-R injury rats, as compared with the SD control rats ( $P < 0.05$ ), but Ad-GABA<sub>A</sub>R treatment significantly reduced the plasma Ang II levels in the GI-R injury rats, as compared with Ad-Con treatment ( $P < 0.05$ ; Fig. 4A). The plasma NE levels, an indication of sympathetic activity (10), were significantly increased in the GI-R rats, as compared with the normal SD rats ( $P < 0.05$ ), although this effect was significantly normalized by Ad-GABA<sub>A</sub>R treatment ( $P < 0.05$ ; Fig. 4B).

**Effects of Ad-GABA<sub>A</sub>R expression on amino acid release.** To investigate whether GI-R expression may result in amino acid change in the LHA, Ad-GABA<sub>A</sub>R was microinjected into the LHA in the GI-R and SD control rats, and amino acid release was assessed. The amino acids examined in

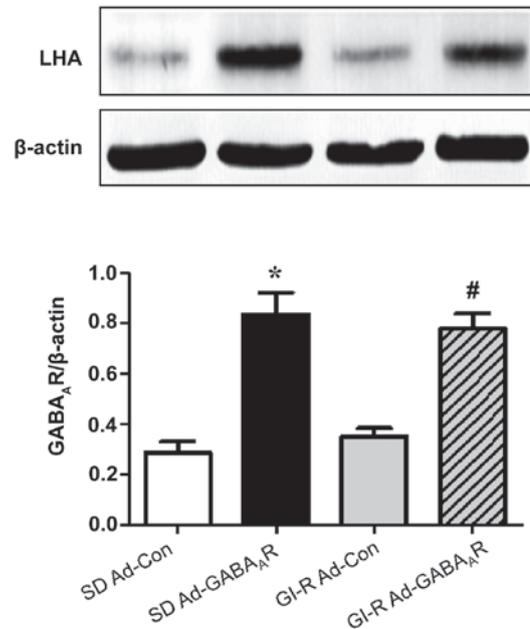


Figure 3. Expression levels of GABA<sub>A</sub>R in the LHA. The data were normalized to β-actin expression levels. \* $P < 0.05$ , as compared with the SD Ad-Con group. # $P < 0.05$ , as compared with the GI-R Ad-Con group ( $n = 4$  in each group). GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; LHA, lateral hypothalamic area; SD, Sprague Dawley; Ad-Con, control; GI-R, gastrointestinal ischemia-reperfusion.

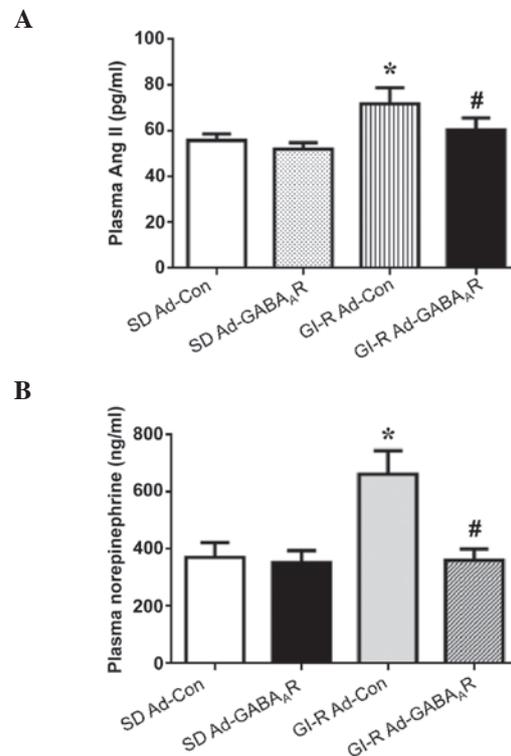


Figure 4. Plasma Ang II and norepinephrine (NE) measurement following Ad-GABA<sub>A</sub>R microinjection;  $n = 8$  for plasma Ang II and NE measurement in each group. (A) Ad-GABA<sub>A</sub>R treatment significantly reduced the plasma Ang II levels. (B) Recombinant adenoviral vectors encoding GABA<sub>A</sub>R significantly increased the expression levels of GABA<sub>A</sub>R in the LHA \* $P < 0.05$ , as compared with the control SD rats. # $P < 0.05$ , as compared with GI-R rats subjected to Ad-Con treatment. Values are mean ± standard error of the mean. Ang II, Angiotensin II; Ad-GABA<sub>A</sub>R, adenoviral vectors overexpressing GABA<sub>A</sub> receptor; SD, Sprague Dawley; GI-R, gastrointestinal ischemia-reperfusion; Ad-Con, control adenoviral vector.

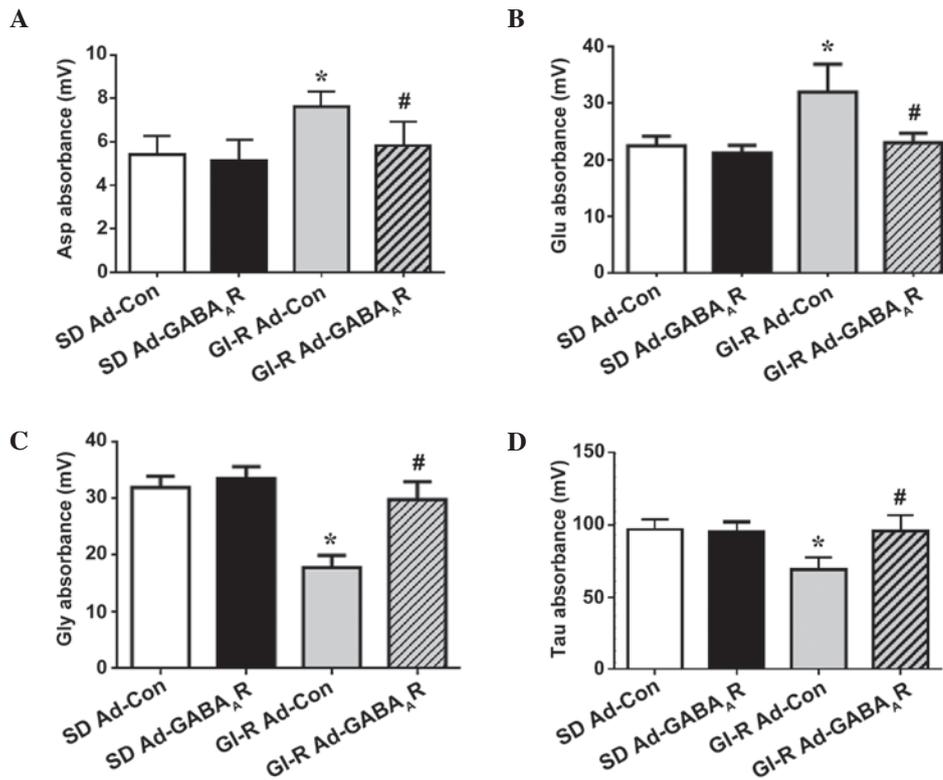


Figure 5. Effects of microinjection of Ad-GABA<sub>A</sub>R into the lateral hypothalamic area on the local release of amino acids. (A) Aspartate (Asp); (B) glutamate; (C) glycine (Gly); (D) taurine (Tau). \*P<0.05, as compared with the control SD rats. #P<0.05, as compared with GI-R rats subjected to Ad-Con treatment. n=8 per group. Ad-GABA<sub>A</sub>R, adenoviral vectors overexpressing GABA<sub>A</sub> receptor; SD, Sprague Dawley; GI-R, gastrointestinal ischemia-reperfusion; Ad-Con, control adenoviral vector.

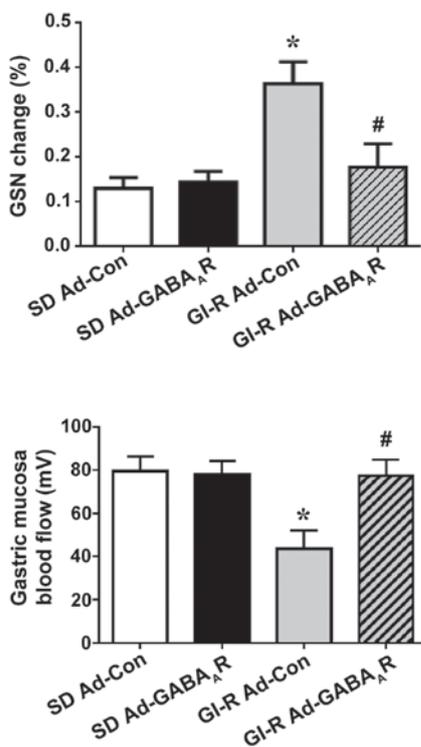


Figure 6. Effects of GABA<sub>A</sub>R on GSN activity and gastric mucosal blood flow. \*P<0.05, as compared with the control SD rats. #P<0.05, as compared with GI-R rats subjected to Ad-Con treatment. n=8 in each group. GABA<sub>A</sub>R, GABA<sub>A</sub> receptor; GSN, greater splanchnic nerve; SD, Sprague Dawley; GI-R, gastrointestinal ischemia-reperfusion; Ad-Con, control adenoviral vector.

the LHA included excitatory [glutamate (Glu) and aspartate (Asp)] as well as inhibitory [taurine (Tau) and glycine (Gly)] amino acids. The baseline release of excitatory amino acid neurotransmitters (Glu and Asp) was significantly increased but that of the inhibitory amino acids (Tau and Gly) was significantly reduced in the GI-R injury rats, as compared with the normal SD rats (all P<0.05). Microinjection of Ad-GABA<sub>A</sub>R into the LHA significantly increased Tau and Gly release, and significantly reduced Glu and Asp release, as compared with Ad-Con microinjection (P<0.05; Fig. 5).

*Effects of GABA<sub>A</sub>R on GSN activity and GMBF.* In order to determine whether the central GABA<sub>A</sub>R mediated GSN activity, the GSN fire frequency was analyzed. The GSN mediates GMBF, which regulates gastric activity. As shown in Fig. 6, microinjection of Ad-GABA<sub>A</sub>R into the LHA following GI-R, significantly reduced GSN activity (P<0.05) and increased GMBF (P<0.05), as compared with Ad-Con treatment.

### Discussion

The present study provides evidence that GABA<sub>A</sub>R overexpression in the LHA results in a profound protection against GI-R injury in rats. In addition to reducing plasma Ang II and NE levels, the LHA-targeted adenovirus reduces the degree of gastric mucosal injury. These results suggest the importance of the GABA<sub>A</sub>R in the LHA in the neural gastrointestinal control and support the hypothesis that GABA<sub>A</sub>R

in the LHA is predominantly involved in the pathophysiological process of GI-R injury (5,11). The present study also demonstrated that an adenovirus, targeting GABA<sub>A</sub>R in the LHA in GI-R rats, effectively inhibited the GSN activity that contributes to the elevated GMBF (12).

An important finding from the present study was that GABA<sub>A</sub>R gene overexpression in the LHA reduced the plasma NE accompanied with improved GI-R injury. One limitation of the present study is that the complex association between over-enhanced GSN activity and the degree of gastric mucosal injury is difficult to clarify. Nevertheless, GSN overdrive has been well-established as a causative factor of GI-R injury, and a close association between GSN activity and GMBF has been identified. Central enhanced GABA signals have been shown to reduce plasma Ang II levels in exercise-training rats (13). In the present study, the plasma Ang II levels were increased in GI-R rats. The adenovirus-induced GABA<sub>A</sub>R overexpression in the LHA normalized plasma Ang II levels in GI-R rats, which may be beneficial for the attenuation of GI-R injury. Furthermore, adenoviruses allow the efficient delivery of relatively large transgenes to the brain, and these viruses infect glial and neuronal cells (14,15). The viruses used in the present study were considered to be specific for GABA<sub>A</sub>R and not for GABA<sub>B</sub>R or GABA<sub>C</sub>R.

The data from the present study support the hypothesis that the GABA<sub>A</sub>R signaling pathway mediates regulative effects on GI-R injury via an increase in excitatory and a reduction in inhibitory amino acid release. Excitatory amino acids (Glu and Asp) induce a GSN tension effect, whereas inhibitory amino acids (Gly and Tau) cause a GSN relaxed response (16,17). In the present study, the release of excitatory amino acids (Glu and Asp) was higher and inhibitory amino acids (Gly and Tau) were lower in the GI-R than in the normal SD control group. Therefore, the GABA<sub>A</sub>Rs in the LHA may regulate gastric activity via the modulation of amino acid release.

In conclusion, in the present study, a GABA<sub>A</sub>R signaling pathway in the LHA during the development of GI-R injury was investigated. Furthermore, the protective effects of GABA<sub>A</sub>R overexpression in the LHA against GI-R injury in rats were analyzed and an increased inhibitory and suppressed excitatory amino acid release was identified.

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