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Effects of γ-aminobutyric acid on aggressive behaviour, jejunum villus morphology, serum biochemical indicators and hippocampal neuropeptide mRNA levels in piglets at weaning with mixing

*Hongyuan Mei, Chengying Yang, Qing Xie, Yang Yang, Xianmei Luo, Hanwei Jiao, Ling Gan**

Department of Veterinary Medicine, College of Animal Science, Southwest University, Chongqing, P.R. China

**Corresponding author: gl9089@swu.edu.cn*

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Abstract: The effects of γ-aminobutyric acid (GABA) on weanling piglets after mixing stress were investigated and the underlying molecular mechanism was analyzed. Sixty weaning piglets were randomly assigned to either the control group (weaning and mixing with a 3 : 3 sex ratio) or the GABA supplement group (30 mg GABA/kg body weight/day + weaning and mixing with a 3 : 3 sex ratio). Aggressive behaviours have been recorded for 2 days and the number of lesions for 3 days. The diarrhea rate on day 6 post-weaning and mixing was analyzed. Serum biochemical indicators, antioxidant variables, jejunum villus morphology and mRNA levels of stress-related neuropeptide genes of the hippocampus were investigated. The GABA addition decreased serum adrenocorticotropic hormone concentrations ($P < 0.05$), aggressive behaviours of weaned piglets 5 h after mixing ($P < 0.05$), lesion scores over the entire 3-day period ($P < 0.01$) and diarrhea rate ($P < 0.01$) and improved jejunum villus integrity. Serum neuropeptide Y (NPY) concentration ($P < 0.05$) and total superoxide dismutase activity ($P < 0.01$) were increased in the GABA supplement group, whereas serum malondialdehyde concentration had a decreasing tendency ($0.05 < P < 0.1$), and glutathione peroxidase activity had an increasing tendency ($0.05 < P < 0.1$). The GABA treatment group had increased mRNA levels of *NPY* ($P < 0.05$) and peptide YY (*PYY*) ($P < 0.05$) in the hippocampus, which may contribute to insights into the regulatory mechanism of GABA in weaning and mixing stress. The addition of GABA is beneficial to reduce weaning and mixing stress in piglets, and NPY and PYY may mediate the process.

Keywords: weaning and mixing stress; aggression; oxidation resistance; villus integrity; growth; neuropeptide Y

Welfare and production issues for farm animals have always been a public concern. After weaning, piglets encounter abrupt separation from the sow, dietary changes, movement to a strange environment, and regrouping into the new litters, which are all powerful stressors (Groot et al. 2001). The

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response to these stressors can be measured by alterations in some behavioural, physiological, neurological, and biochemical indicators as well as gene expression studies in the brain (Tuchscherer et al. 2004). Among those changes, aggressive behaviours are good indicators of the stress level induced by social isolation (Grippo et al. 2007). Post-weaning mixing and regrouping of unfamiliar piglets results in vigorous aggressive behaviours and decreased growth (Mei et al. 2016), and these stressors have relatively long-lasting effects such as the occurrence of pawing, belly-nosing and other abnormal behaviours for at least 3 weeks (Van Putten and Dammers 1976). Mixing at a 3 : 3 sex ratio at weaning was reported to produce the most aggressive behaviours (Mei et al. 2016).

Adrenocorticotropic hormone (ACTH) is a hormone that is often measured and reported as an indicator of acute stress. Investigations of adrenocortical responses to weaning and mixing have found increased cortisol concentration within the first week after weaning and mixing (Kanitz et al.1998). Elevated levels of cortisol, if prolonged, can lead to proteolysis and muscle wasting (Simmons et al. 1984). Therefore, the examination of ACTH, cortisol and blood urea nitrogen (BUN) levels provides important information about stress responses. Furthermore, stressors also increase reactive oxygen species (ROS) in the body, which may trigger oxidative injury through lipid peroxidation and protein oxidation and stimulate toxic product synthesis and cell death, whereas serum malondialdehyde (MDA) content, total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) activity are good biochemical indicators of stress responses in piglets (Armstrong and Browne 1994).

The weaning and mixing of piglets are both stressful events that change intestinal morphology and gastrointestinal function, and they can produce diarrhea (Boudry et al. 2004). Growing evidence shows that γ-aminobutyric acid (GABA) plays a large role in increasing voluntary food intake (VFI), regulating endocrine secretion, improving nutrition utilization and alleviating stress in mammals (Tajalli et al. 2006). Brain levels of GABA are low in mice and rats that exhibit aggressive behaviours, which have been interpreted as being concordant with the proposed inhibitory role of GABA in aggression (Clement et al. 1987). Furthermore, supplementation with GABA also improved antioxidation activity in heat-stressed Roman hens (Zhang et al. 2012) and increased the activity of superoxide dismutase (SOD) in transition dairy cows (Wang et al. 2013). These observations led us to hypothesize that GABA can regulate stress, which is reflected in reducing aggressive behaviours and improving jejunum villus morphology and antioxidation activity in weanling and mixing piglets.

The GABAergic system is widely distributed in the hippocampus of piglets and has an inhibitory action on the neurons in the hippocampus. The hippocampus has been reported to be sensitive to monitoring the physiological environment and modulating an appropriate response, which is mediated by a variety of neuropeptides rich in the hippocampus (Lathe 2001). Of these, serum neuropeptide Y (NPY) is widely distributed in the CNS and is involved in the cerebral regulation of food intake, cognition, anxiety, mood and stress resilience (Heilig 2004; Morales-Medina et al. 2010). Furthermore, six neuropeptides, including peptide YY (PYY), hypocretin neuropeptide precursor (HCRT), neuropeptide S (NPS), neuromedin U (NMU), proenkephalin (PENK) and galanin (GAL), in the hippocampus of the piglets are suggested to be involved in the stress responses of piglets exposed to iron dextran (Gan et al. 2016). Therefore, we want to know whether or not these neuropeptides also mediate the anti-stress effect of GABA on weaning and mixing stress in piglets. Profiling the change in expression levels of neuropeptide genes in the hippocampi of weaning and mixing piglets will be helpful and provide insights into the stress regulation mechanisms of GABA.

To our knowledge, limited research has been done on GABA supplementation in piglets under weaning and mixing stress conditions. The objective of this study is to determine the effects of GABA on aggressive behaviours, jejunum villus morphology, and serum biochemical indicators and to preliminarily explore whether these neuropeptides mediate the effect of GABA on mixing and weaning stress in piglets by investigating the changed transcription levels of some neuropeptide genes involved in stress regulation in the hippocampus.

MATERIAL AND METHODS

Animals, feeding, grouping and treating. Ten litters of crossbred Landrace × Yorkshire dams ×

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Duroc sire with similar initial weights were the experimental piglets used in this study. These piglets were born from 10 fourth-parity sows at a standard commercial farm, Ju Wang, in Chongqing, China. The experimental litters were initially selected to ensure that the litter size was greater than eight, with more than 3 male or female piglets. The piglets were kept with their mothers in farrowing pens measuring 2.1 × 2.0 m, with a slatted floor and wooden kennels. The kennels (1.0 × 0.6 m, 0.6 m high) were in one corner of the pen with a heat lamp (22°C), straw (1 kg per pen) and a piglet feeder for the duration of lactation. Six piglets, including 3 barrows and 3 females from each litter, were randomly chosen and marked with a marker after excluding piglets that were underweight (less than 3 kg), overweight (more than 9 kg) or unhealthy on day 21 of age. The pen (litter) with marked piglets was randomly divided into either the GABA (purity > 98%; Sangon Biotech, China) supplement group (weaning and mixing with 3 : 3 sex ratio + 30 mg/kg body weight (BW)/day) or the control group (weaning and mixing with 3 : 3 sex ratio). Each treatment was replicated with five pens of six piglets (3 barrows and 3 females) per pen. Taking into account the suggested levels from Tajalli et al. (2006) and Yang et al. (2009), the piglets in the treatment group were orally administered GABA (30 mg/kg BW/day) dissolved in saline starting one week before weaning and ending 6 days after weaning. The piglets in the control group were administered the same dose of saline.

The 30 piglets in the GABA supplement group or the control group were weaned in the maternity room and regrouped into 5 new post-weaning pens during the mixing of piglets from the two different litters with balanced weaning weights. There were no significant differences in weaning piglet weight (control group: 6.56 ± 0.19 kg, treatment group: 7.01 ± 0.16 kg, $P > 0.05$) at 28 days of age. The 5 litters of the GABA supplement group and the 5 litters of the control group were mixed separately, with both placed with 3 : 3 mixing ratio. In the mixing process, 3 barrows from one litter were always mixed with 3 females from the other litter to avoid sex differences. Each replicate consisted of 6 piglets (3 barrows, 3 females) from two different litters. Each of the piglets was identified by labelling numbers (from 1 to 6) on their left and right abdomen with a non-toxic black marker. Weaning was carried out between 10:00 and 14:00 h. All experimental piglets were kept in the same environmental conditions, with an indoor temperature of 22 ± 2°C and a 12 : 12h light/dark cycle. During the entire experimental process, the animals were given *ad libitum* access to food and water. Enough feeding space (0.70 m^2 per piglet) was provided. No prophylactic antibiotic treatment was administered during the experimental period. The diet was formulated as suggested by National Research Council (2012) (Supplementary Table S1 in Supplementary Online Material (SOM). All the housing and management of animals was conducted following standard commercial practices including clipping teeth, tail docking, ear notching and castrating males on day 15 after birth. The castration was performed using the National Pork Board's (1996) approved method. The same animal caretaker performed all these interventions.

Behavioural observations and lesion scores. The behaviours of the piglets on the first 2 days after mixing and regrouping (day 1: 5 h, day 2: 6 h) were continuously observed and recorded by a monitor that was positioned at a height of 2.3 m and that had a bird's eye view of the entire pen (Melotti et al. 2011). The definitions of the observed behaviours are listed in Supplementary Table S2 in SOM. The observed data for the first 5 h and a one-hour scan sampling (starting at 8:00 h, 10:00 h, 12:00 h, 14:00 h, 16:00 h and 18:00 h) on the second day were collected and analyzed to evaluate piglet aggression.

The total number of lesions was assessed at 5 h (day 1), on the second day (day 2) and on the third day (day 3) after mixing and regrouping. The skin injury scores were recorded and analyzed using the procedure described in Melotti et al. (2011) and Mei et al. (2016).

Diarrhea rate. Faeces were observed from weaning to day 6 post-weaning. Trained observers blind to the treatments visually assessed fecal consistency at 9:30 h and 16:30 h each day. Fresh feces were graded according to the following scale: 1 = hard feces; 2 = slightly soft feces; 3 = soft, partially formed feces, 4 = loose, semiliquid feces; and 5 = watery, mucous-like feces. Recurring fecal scores of 4 or 5 for 2 consecutive days was defined as diarrhea. The diarrhea rate was calculated according to the following formulas:

$$\text{diarrhea rate (\%)} = (\text{number of pigs with diarrhea} \times \text{diarrhea days}) / (\text{number of pigs} \times \text{total observational days}) \times 100$$

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Tissue collection. After 2 days of mixing and regrouping, one barrow close to the average group body weight of barrows from each replicate was selected and fasted for the whole night and was then anesthetized with chloral hydrate (10%) (Damao Chemical Reagent Factory, China) and decapitated the next morning. The blood in the precaval vein was collected and allowed to clot for 30 min before the serum was centrifuged (3000 rpm/min, 10 min) at 4°C and collected for further analysis. Approximate middle segments of the jejunum (0.2 × 0.5 cm) were collected and fixed in 2.5% glutaraldehyde fixation fluid for further analysis of the intestinal morphology by transmission electron microscopy (TEM). After piglets were decapitated, the skull was immediately opened using the electric motor saw and operating forceps, the brain was removed, and hemisected along the longitudinal fissure, and each hemisphere was prepared for sampling. Immediately thereafter, samples were collected, frozen in liquid nitrogen and stored at –80°C until analysis. The experimental procedures followed the laws of animal protection and were approved by the Animal Care Advisory Committee of Southwest University, China.

Serum analyses. An ultraviolet spectrophotometer was used to detect serum GSH-Px activity (dithio-nitrobenzoic acid method), BUN concentration (urease method), MDA concentration (TBA method) and T-SOD activity (hydroxylamine method). ELISA was used to test NPY concentration (ELISA method), ACTH concentration (ELISA method) and cortisol concentration (ELISA method). The coefficient of variation of intra-assay and inter-assay ELISA kits was less than12% and 10%, respectively. All kits were used according to the manufacturer's instructions (Nanjing Jiancheng Bio Company, China).

Transmission electron microscopy. Jejunum blocks were fixed in 30% glutaraldehyde in 0.1 M sodium cacodylate buffer at a pH of 7.2 for 2 h. The specimens were post-fixed in 1% aqueous osmium tetroxide for 45 min after washing in buffer solution. Then, the tissue blocks were embedded in araldite followed by dehydration in acetone. Sections 1 μm thick were observed microscopically before ultra-thin sections were cut and stained with 0.25% uranyl acetate (Phillips EM 400 transmission electron microscope) at low and high magnification.

Reverse transcription-quantitative real-time PCR (RT-qPCR). The total RNA from the hippocampus was extracted using TRIzol reagent (Invitrogen, USA). The quality of the total RNA

Table 1. Primer sequences

Gene (Accession No.)	F/R	Sequence	Amplicon (bp)	Annealing temperature (°C)
GAL (gi: 47523541)	F	*CAACCACAGATCATTCCACGA*	145	60
	R	*CAAGAAAGCCAGAAACTCCATT*		
HCRT (gi: 134085366)	F	*CCTCAAAGTTCCTGGCTATTC*	98	60
	R	*AGCAGCGTCACGGTGGCCCA*		
NMU (gi: 648945328)	F	*GCACTGGAGGAGCTTTGTCT*	122	60
	R	*GAATTGCCCAACTTTCGTGT*		
NPS (XM_021073718.1)	F	*TCTGATTACTCTGTCATCCTGCTG*	77	60
	R	*GGCTTTAGCAAATCAAGTCCC*		
NPY (gi: 373432748)	F	*CAGATACTACTCGGCGTTGAG*	159	60
	R	*CATCACCACACAGAAGGGTCT*		
PENK (gi: 545821721)	F	*GAGTGGTGGATGGACTATCAGAA*	109	60
	R	*TCTCGGGAACCTCCTTTGA*		
PYY (gi: 374092749)	F	*CTCTGGAGCTGTCGCTATGG*	193	60
	R	*GACCAGGTTGAGGTAGTGGC*		
18S rRNA (gi: 37956930)	F	*TCCGGAATCGAACCCTGAT*	60	60
	R	*GTAGTCGCCGTGCCTACCA*		

GAL = galanin, *HCRT* = hypocretin neuropeptide precursor, *NMU* = neuromedin U, *NPS* = neuropeptide S, *NPY* = neuropeptide Y, *PENK* = proenkephalin, *PYY* = peptide YY, F = forward primer, R = reverse primer

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was determined using SmartSpec Plus (BioRad, USA) as the ratio of absorbance at 260 nm to the absorbance at 280 nm, and the total RNA concentration was measured at a wavelength of 260 nm. The total RNA integrity was examined on a 1% agarose gel, which typically produces two major bands comprising the 28S and 18S ribosomal RNA (rRNA) species and a ratio of 28S/18S indicates RNA of high quality. Then, 100 ng of RNA was treated with DNase I (TaKaRa, China) and allocated to each reverse transcription reaction to synthesize cDNA with a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa). The synthesized cDNA was diluted to 50 µg/µl with sterile water and stored at –20°C until further use as a template. Gene-specific PCR primers were designed for *GAL*, *NPY*, *NMS*, *NMU*, *PENK*, *PYY*, and *HCRT* with Primer 5.0 software. The primer sequences can be found in Table 1. The dissociation curves were examined for each primer set to determine the efficiency of primers (90–110%) for a single target sequence and to choose the set that best amplified only our region of interest. The analyses of the dissociation curves did not reveal any non-specific PCR products. The level of 18S rRNA transcripts was used as the endogenous control, and its level was statistically significantly unaffected by the treatments. Quantitative real-time PCR was performed using SYBR® *Premix Ex Taq*™ (TaKaRa) with Step One™ software (ABI StepOne, USA) to determine cycle threshold (C_t) values. The PCR reaction system (20 µl) contained 10 µl SYBR® *Premix Ex Taq*™, 0.4 µl ROX reference dye, 0.4 µl (10 mM) each of forward and reverse primers, 2 µl cDNA templates, and 6.8 µl RNA-free H_2O. qPCR was performed under the following conditions: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and then a melting curve of 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. For analysis of the C_t values, each sample was run in triplicate, and those triplicates were averaged to assign C_t values for each sample and each gene. Relative quantity was determined using the $\Delta\Delta C_t$ method.

Statistical analysis. A mixed model of SPSS 20.0 (IBM, USA) was used to analyze the data. The GABA treatment was fixed effect, sex and litter were random effects and the initial body weight was covariate to adjust the difference of initial body weight. The Shapiro-Wilk test was conducted to evaluate the normality of the data. Each pen was used as an experimental unit. For the lesion score, two-way ANOVA was used to examine the effects of time and treatment, with variance between groups assessed by Levene's test for quality of variance and post-hoc Tukey's HSD test. Probability values of $P < 0.05$ were used to define statistical significance, and values of $P < 0.1$ and $P > 0.05$ were accepted as statistical trends.

RESULTS

Aggressive behaviours. During the first 5-h observation period, compared to the control group, the latency to start the first fight in the GABA treatment group increased significantly ($P < 0.05$), and the number of fights and the proportion of fighting time decreased significantly ($P < 0.05$). The time spent fighting had a significantly decreased tendency ($0.05 < P < 0.1$). However, GABA treatment did not significantly alter the time spent

Table 2. Effect of γ-aminobutyric acid (GABA) on aggressive behaviours of weaned piglets 5 h and on the 2nd day after mixing ($n = 5$)

Item	5 h after mixing				2nd day after mixing			
	Control	GABA	SEM	*P*-value	Control	GABA	SEM	*P*-value
Latency to first fight (s)	156.40	787.00*	149.43	0.017				
No. of fights	24.40	12.20*	3.68	0.047	5.20	4.40	1.73	0.752
Time spent fighting (s)	1019.60	308.60*	200.35	0.036	191.75	141.75	57.10	0.559
Proportion of fighting time (%)	5.67	1.71*	1.11	0.036	0.85	0.63	0.309	0.625
Time spent bullying (s)	185.40	122.20	31.97	0.200	66.60	64.80	25.87	0.962
No. of bullying	21.00	16.60	5.46	0.585	8.80	6.40	2.07	0.437

piglets' behaviour during the first 2 days after mixing and regrouping (day 1: 5 h, day 2: 6 h) was continuously observed and recorded; asterisks indicate significant differences from controls: *$P < 0.05$

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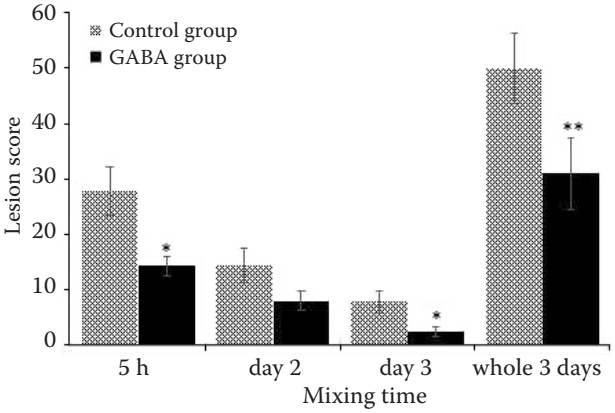

Figure 1. Effects of GABA on the lesion score of weaned and mixing piglets at 5 h, on day 2, day 3 and the whole three days after weaning and mixing; asterisks indicate differences from control: $^{*}P < 0.05$, $^{**}P < 0.01$

bullying ($P > 0.05$) or the number of bullied piglets ($P > 0.05$). Additionally, there was no significant difference in the aggressive behaviours between the control group and the GABA treatment group on the second day after mixing ($P > 0.05$) (Table 2).

Lesion score. A significant difference was found between the control group and the GABA treatment group in lesion score at 5 h ($P < 0.05$), day 3 ($P < 0.05$) and over the entire 3-day period ($P < 0.01$) (Figure 1), and there was no significant difference on the second day after mixing. The results from a two-way ANOVA showed that there was homogeneity of variance between groups, as assessed by Levene's test for quality of variances. There was no significant interaction between the effect of day and treatment on lesion score ($P > 0.05$).

Table 3. Effects of γ-aminobutyric acid (GABA) on the serum indices of piglets ($n = 5$)

Item	Control	GABA	SEM	*P*-value
MDA (nmol/ml)	2.56	2.16	0.11	0.063
T-SOD (U/ml)	61.96	73.88**	1.69	0.008
GSHPx (U/ml)	337.67	443	38.41	0.083
BUN (mg/l)	274.89	200.85	26.27	0.117
ACTH (ng/l)	756.02	642.12*	24.92	0.045
NPY (ng/l)	682.32	1142.76*	57.86	0.015
Cortisol (ng/l)	445.51	380.06	23.60	0.121

MDA = malondialdehyde, T-SOD = total superoxide dismutase, GSH-Px = glutathione peroxidase, BUN = urea nitrogen, ACTH = adrenocorticotropic hormone, NPY = neuropeptide Y; asterisks indicate significant differences from controls: $^{*}P < 0.05$, $^{**}P < 0.01$

Serum indices. The effects of treatment on serum variables are presented in Table 3. Compared with the control group, the serum SOD and NPY concentration of piglets supplemented with GABA significantly increased ($P < 0.01$, $P < 0.05$), and the ACTH concentration significantly decreased ($P < 0.01$). The MDA concentration had a decreasing tendency ($0.05 < P < 0.1$), while the GSH-Px activity had an increasing tendency ($0.05 < P < 0.1$). Serum BUN concentration and cortisol concentration were not altered by GABA treatment.

Diarrhea rate. As shown in Table 4, the diarrhea rate significantly decreased compared with that of the control group ($P < 0.01$).

Jejunum villus morphology. The jejunum villi of weaned piglets in the control group were rare

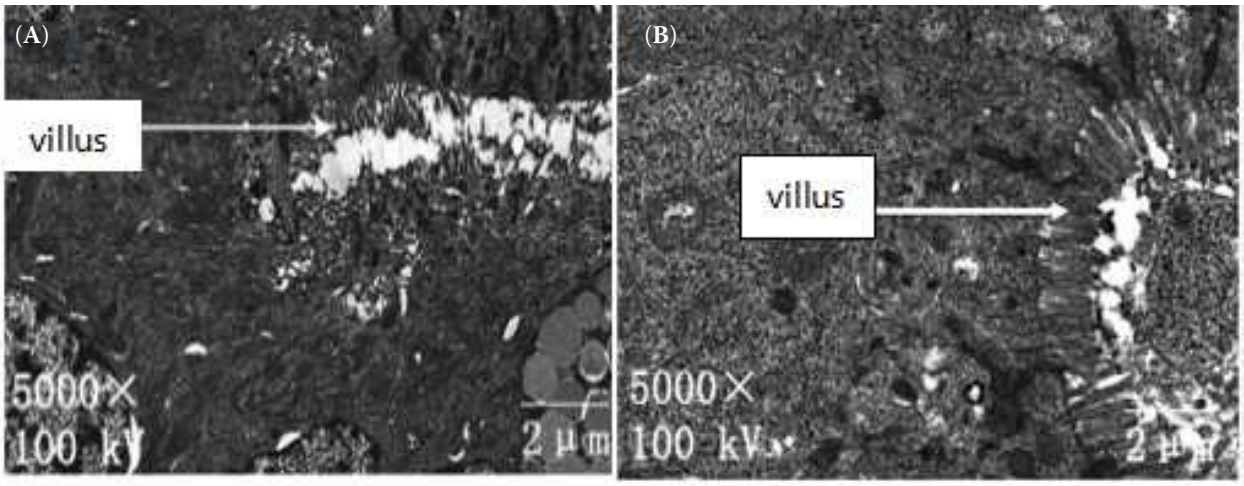

Figure 2. Effects of GABA on jejunum villus morphology of weaned and mixing piglets: (**A**) control group, (**B**) GABA treatment group

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Table 4. Effects of γ-aminobutyric acid (GABA) on diarrhea rate in piglets for initial six days after weaning and mixing (n = 5)

Item	Control	GABA	SEM	P-value
Diarrhea rate (%)	23.33	5.56**	2.89	0.002

asterisks indicate significant differences from controls: *P < 0.05, **P < 0.01

and spread out, while the jejunum villi of weaned piglets in the GABA treatment group were densely packed and neatly arranged (Figure 2).

mRNA levels of neuropeptide genes. Real-time PCR was used to detect the effect of GABA on seven neuropeptide genes associated with stress regulation in the piglet hippocampus. The results showed that GABA significantly increased mRNA levels of the *NPY* (P < 0.05) and *PPY* (P < 0.01) genes in the hippocampus (Figure 3).

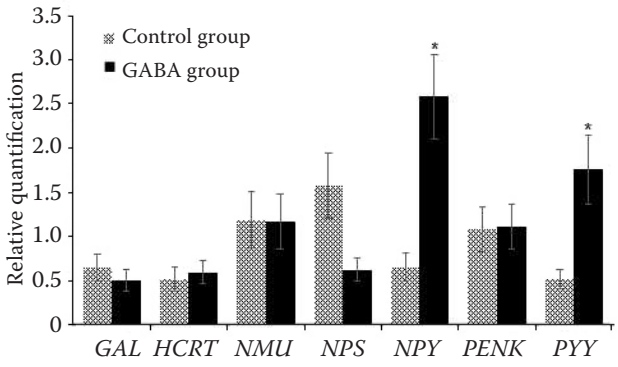

Figure 3. Effects of GABA on mRNA levels of seven neuropeptide genes in the hippocampi of weaned and mixing piglets (n = 5)

asterisks indicate differences from control: *P < 0.05

DISCUSSION

Our previous study indicated that weaning and mixing with a 3 : 3 sex ratio is a powerful stressor for piglets that induced vigorous aggressive behaviours (Mei et al. 2016). In the study, the significantly reduced aggressive behaviours and lesion scores of weaning and mixing piglets fed a GABA supplement suggest that GABA can improve reactions to stress induced by weaning and mixing. Furthermore, the ACTH concentration in piglets' serum was significantly decreased by the GABA supplement, further indicating the weakened stress level, which was similar to the effect of the GABA supplement on heat-stressed Roman hens (Zhang et al. 2012) and beak trimming stressed chicks (Xie et al. 2013).

Stress-induced alteration of intestinal flora, anorexia and weight loss are striking since piglets consume more energy to support aggressive behaviours such as fighting. Conversely, excessive aggression aggravates stress, diarrhoea, exhaustion and skin lesions (Stookey and Gonyou 1994; Boudry et al. 2004). In the present study, GABA treatment made the jejunum villi of piglets more densely packed and neatly arranged compared to the control group, which also contributes to the significantly decreased diarrhea rate.

Furthermore, the decreased ACTH concentration in the GABA supplementation group contributed to the improved antioxidant status of the piglets. The GSH-Px can convert H_2O_2 induced by stressors into less-dangerous reduced forms. SOD plays an important role in antioxidant defense mechanisms in animals. MDA is a hydrolysis product of lipid peroxidation, and the increased MDA content is considered an indicator of oxidative stress (Armstrong and Browne 1994). Previous evidence indicated that antioxidant enzyme activities are lower in stressed piglets, which, in turn, exacerbates the existing oxidative stress (Yang et al. 2017). In this study, the decreased trend of MDA, the increased trend of GSH-Px and the significantly increased serum T-SOD content indicated enhanced antioxidant status in the piglets treated with GABA, which also demonstrated that GABA resisted the oxidative stress induced by weaning and mixing, consistent with the results observed in rats (Baydas et al. 2005).

Moreover, weaning and mixing-induced oxidative stress was reported to induce intestinal epithelial cell apoptosis and affect the intestinal tissue of piglets by inhibiting nucleotide-excision repair (NER) *in vivo*, and an antioxidant-rich diet could compensate for this effect (Boudry et al. 2004; Cai et al. 2016). Therefore, the improved antioxidant enzymes from the GABA supplement may be involved in inhibiting intestinal epithelial cell apoptosis and protecting NER against oxidative stress-induced inhibition along with the improvement in villus integrity, which made GABA supplement significantly reduce the diarrhea rate of piglets.

The role of NPY and PYY in the behavioural manifestation of immune challenge is suggested by many properties of this peptide family (Kormos

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and Gaszner 2013). In the study, the significantly increased levels of the *NPY* and *PYY* transcripts suggested mediation in the hippocampus through stress regulation by GABA. NPY has many functions, and two important ones are known as important components of stress antagonizing peptides that regulate food intake by mammals (Pu et al. 1999; Heilig 2004). Peptide knockout experiments also indicate that NPY and PYY stimulate food intake and resist the stimulation of Bacille Calmette–Guérin (BCG) in mice (Painsipp et al. 2008). Therefore, we speculated that the NPY and PYY signaling systems in the hippocampus possibly mediated the role of GABA in the anti-stress process.

CONCLUSION

Supplementation with GABA can improve the stress response of weaning and mixing piglets, resulting in reduced aggressive behaviours, decreased lesion scores, increased NPY concentrations, improved antioxidative abilities and jejunum villus morphology. The significantly increased *NPY* and *PYY* mRNA levels in the piglet hippocampus may mediate the beneficial effect of GABA on stress in weaning and mixing piglets.

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