Research Article

Effects of Parabolic Flight on Serotonin-Related Gene Expression in the Mouse Brain

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ABSTRACT

Exposure to uncontrollable stress triggers a wide range of adaptive changes in the central nervous system (CNS), including the elevation of serotonin metabolism and an increased susceptibility to affective disorders. The present experiment investigated alterations in gene expression levels, especially serotonin-related genes, in mouse brains exposed to gravity-changing stress using a competitive reverse transcription-polymerase chain (RT-PCR) reaction. Mice were exposed to gravity-changing stress during eight parabolic flights. Serotonin and tryptophan transporters and tryptophan hydroxylase 2 mRNA levels in the midbrain were significantly increased 6 hr after the flight compared to pre-parabolic flight controls. In contrast, the 5-HT1A receptor, GAD65/67, and tyrosine hydroxylase mRNAs were not altered by the flight. These results suggest that the serotonergic system, particularly presynaptic synthetic pathways, were activated in the CNS by gravity-changing stress.

Key words: Animals; Stress; Gene Expression; Monoamines; GABA; Microgravity

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INTRODUCTION

Stress induces alterations in serotonin-related functions (Chaouloff, 1993), and exposure to repeated uncontrollable stress leads to psychopathologies, such as anxiety disorders and depression (McEwen, 1998; Kendler et al., 1999). The role of serotonin in stress-related psychopathologies is highlighted by the successful use of serotonin-modulating drugs for the treatment of clinical conditions, such as generalized anxiety, panic disorder, obsessive compulsive disorder, and depression (Graeff et al., 1996; Graeff, 2002). Several psychological and physiological stresses in animal models, including electric shock, immobilization, and inescapable sound, increase serotonin turnover, as measured by an elevation in the 5-hydroxyindole

acetic acid to serotonin ratio, in many brain areas that are innervated by serotonergic neurons (Inoue et al., 1994; Kawahara et al., 1993; Kirby et al., 1997). Repeated immobilization stress also alters the enzymatic activity of tryptophan hydroxylase (TPH), which is the rate-limiting enzyme in serotonin biosynthesis (Culman et al., 1984), and TPH mRNA levels (Chamas, 1999) in the raphe nuclei. An adaptive response to emotional stress in the serotonergic system follows the perception of dangerous and/or unpredictable situations (Yoshioka et al., 1995).

The alterations in CNS function that are observed during parabolic flights are not solely due to the repeated gravity changes that are experienced during the flight. However, these changes may be related to secondary psychophysiological reactions to the emotional and physical stress during these flights. The present experiment investigated alterations in gene expression levels, especially serotonin-related genes, in mouse brains exposed to gravity-changing stress. We determined the expression of mRNA for tryptophan hydroxylase (TPH) genes 1 and 2, tryptophan transporter (LAT1), the serotonin transporter (SERT), monoamine oxidase (MAO) genes A and B, and the serotonin-1A (5-HT1A) receptor using the quantitative reverse transcription-polymerase chain reaction (RT-PCR) in mice exposed to parabolic flight. γ-Aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the mammalian brain, is synthesized by two glutamic acid decarboxylase (GAD) isoforms, GAD65 and GAD67. These GABA-related genes were also quantified. This strategy is novel for psychological adjustment and adaptation research, and it may be extrapolated to patients with anxiety disorders, especially panic disorder.

Serotonin-related gene expression in the small intestine was also examined in this experiment as a positive control because serotonin is synthesized in gastrointestinal tissues as a local hormone.

MATERIALS AND METHODS

Animals

All studies involved naïve, male C57BLK/6J mice that were obtained from the SLC (Hamamatsu, Japan) at 8 weeks of age. The mice were housed at a constant temperature (23°C) and humidity (40 - 60%) on a 12-hr light/dark cycle. Food and water were provided *ad libitum*. The Animal Care and Use Committee of Hokkaido University Graduate School of Medicine approved this experiment.

Parabolic Flight Experiment

During the flight, eight parabolas were flown within a 1-hr time period. Each parabola included changes in gravity (1.4 G, 0 G, and 1.4 G), and each gravity phase lasted approximately 40 sec, 15 sec, and 40 sec, respectively (Figure 1). A Mitsubishi MU-300 aircraft operated by Diamond Air Service Co. (Nagoya, Japan) was utilized for the parabolic flight experiment (Yoshioka et al., 2010).

Tissue Preparations

Midbrains, including raphe nuclei (dorsal and median raphe nucleus), and small intestines containing enterochromaffin cells were dissected 6 hr after the parabolic flight. Control tissues were also obtained on the aircraft just prior to the first parabolic flight.

Analysis of mRNAs for Serotonin-Related Genes using Quantitative RT-PCR

Total RNAs were isolated from each sample of brain and small intestine using RNAiso® (TaKaRa Baio, Japan) following digestion with RNase-free DNase I. Total RNA (4 μg) was used for cDNA synthesis with oligo(dT) primer, random hexamers, and PrimeScript® RT Enzyme Mix I. The solution was heated for 5 sec at 85°C and incubated for 15 min at 37°C. Quantitative real-time PCR was performed in a Thermal Cycler Dice Real Time system (TaKaRa Bio, Shiga, Japan) using SYBR Premix Ex Taq II ® (TaKaRa Bio, Japan). The cycling conditions for all primers were as follows: 30 sec at 95°C to activate the DNA polymerase followed by 40 cycles of two steps, 5 sec at 95°C (denaturation) and 30 sec at 60°C (annealing extension). Each mRNA level was evaluated by comparison with a house-keeping gene, HA067799 (18S ribosomal RNA). The primers used in this experiment are listed in Table 1. We measured GABAergic-related and serotonergic genes.

Determination of Serum Corticosterone

Blood was sampled prior to and 6 hr after exposure to parabolic flight. Samples were separated using a centrifugal separator (10,000 g for 10 min), and serum was stored at -80°C until analysis. Serum corticosterone levels were measured in duplicate using enzyme immunoassay (ELISA) kit (Assaypro, Winfield, MO, USA).

Statistical Analysis

Values are presented as means ± standard errors. Statistical analysis was performed using unpaired *t*-tests for RT-PCR experiments and one-way ANOVA followed by Bonferroni post hoc tests for corticosterone measurements. Statistical significance was considered at $p < 0.05$.

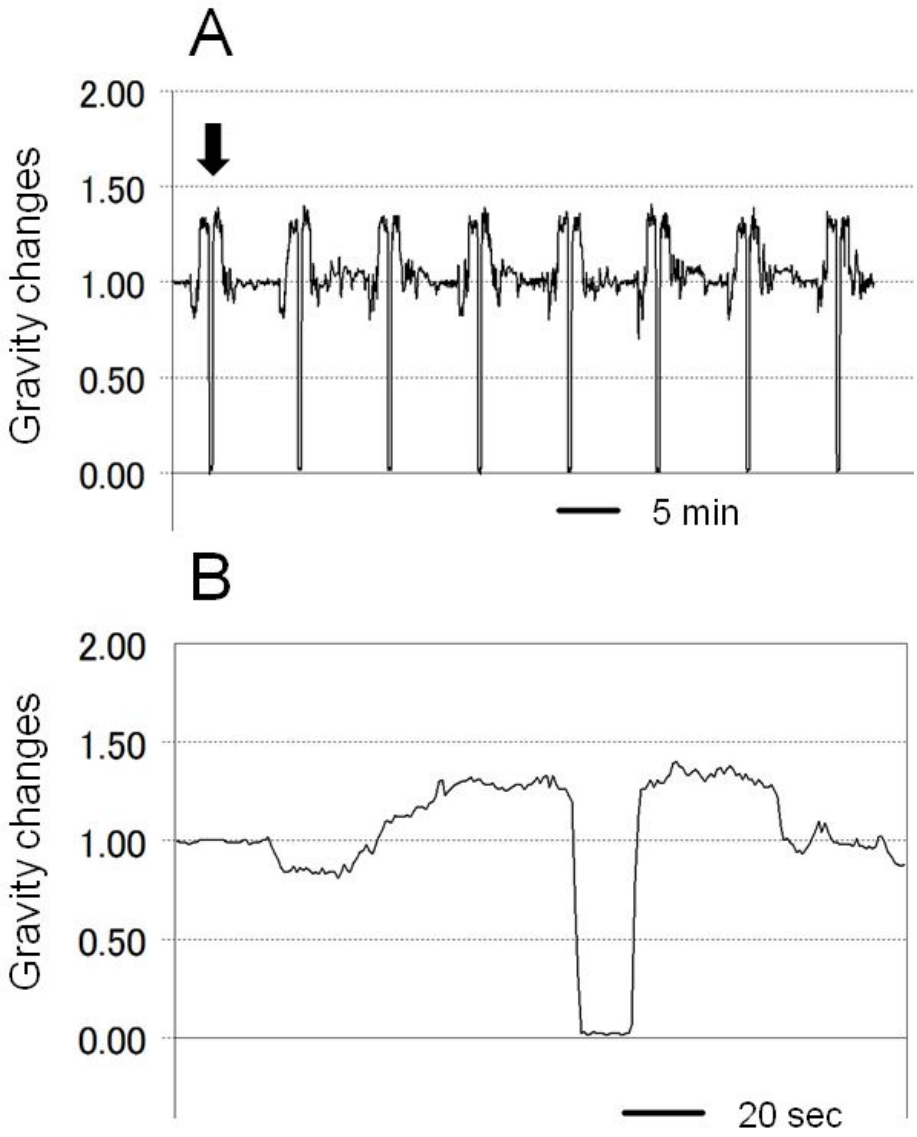

Figure 1. Representative changes in gravity during parabolic flights. Eight parabolas were performed in this experiment (A). Each parabola provided 3 x 10^{-2} G for 15 sec and 1.4 G for approximately 40 sec prior to the parabolic flight. (B) illustrates a specimen record from the first parabola. The y-Axis indicates changes in gravity. A Mitsubishi MU-300 aircraft operated by Diamond Air Service Co. (Nagoya, Japan) was used for the parabolic flight experiment.

Table 1. Real-Time PCR Primers.

Gene Symbol	GenBank Acc.	Forward Primers	Reverse Primers	Product Size (bp)
TPH1	NM_009414	5'CATCACGAGCTTCCAGGATGTCTA3'	5'TGAACACTCTGTGTGTACGGGTTG3'	2047
TPH2	NM_173391	5'GACCCTGAATCCGCCTGAGA3'	5'ACATGAGGACTCGGTGAGAGCA3'	1649
MAOB	NM_172778	5'TTGGCAGCCAGAACCAGAATC3'	5'TGGTGGTCAATCCAAACAGCTTTA3'	2410
SERT	NM_010484	5'CACGCTGGGTTTGGATAGCA3'	5'CCACGATGAGCACAAACCATTC3'	2744
LAT1	NM_011404	5'ATGGAGTGTGGCATTGGCTTC3	5'GCATCAACTTCTGGCAGAGCA3'	3535
MAOA	NM_173740.2	5'GCCCAGTATCACAGGCCACA3'	5'TCTATCCCGGGCTTCCAGAAC3'	4068
5HTR1A	NM_008308.4	5'CGCTGATCTCGCTCACTTGG3'	5'TGCTGATGGTGCACTCGTTG3'	4441
TH	NM_009377.1	5'CCGCACATTTGCCCAGTTC3'	5'TGCACCGTAAGCCTTCAGCTC3'	1757
GAD65	NM_008077.4	5'TTCTGGTACATTCCACAAAGCCTTC3'	5'CCATGGTTGTTCCTGACTCCATC3'	3231
GAD67	NM_008078.2	5'TACAGCAGCTCTGCCATCCAC3'	5'GGGATTTGGACCAACGTTCAATA3'	5625

TPH, tryptophan hydroxylase; MAO, monoamine oxidase; SERT, serotonin transporter; LAT, L-type amino acid transporter; 5HTR1A, serotonin 1A receptor; TH, tyrosine hydroxylase; GAD, glutamic acid decarboxylase

RESULTS

Expression of mRNA in the Midbrain and Small Intestine

We explored the impact of parabolic flight on gene related serotonin synthesis and neurotransmission in the midbrain containing the raphe nuclei. Real-time PCR revealed that parabolic flight increased TPH1 and TPH2 mRNA levels by approximately 1.5- and 2.0-fold, respectively (Figure 2). LAT1, SERT, and MAO-B mRNA levels were also increased by parabolic flight (Figure 2). TPH1 and TPH2 are the rate-limiting enzymes in serotonin synthesis, and LAT1 and SERT play significant roles in *de novo* serotonin synthesis and its transport, respectively. Serotonin is metabolized by MAO-A but not MAO-B. The primary serotonin receptor in the brain for anxiety and fear is the 5-HT1A receptor. Parabolic flight markedly influenced serotonin-related genes, especially genes for presynaptic serotonin mobilization and recruitment, in the midbrain but not in the receptor site. Serotonin–related gene expression in the small intestine was not altered by parabolic flight.

Figure 3 illustrates the effects of parabolic flight on the mRNA expression of other stress-related neurotransmitter synthases. GAD65 and GAD67 synthesize GABA in the brain, and tyrosine hydroxylase (TH) is the rate-limiting enzyme for catecholamine synthesis. The mRNA expression of these enzymes was not significantly altered.

Serum Corticosterone

Parabolic flight significantly increased serum corticosterone levels. Corticosterone levels returned to control levels 6 hr after parabolic flight (Figure 4).

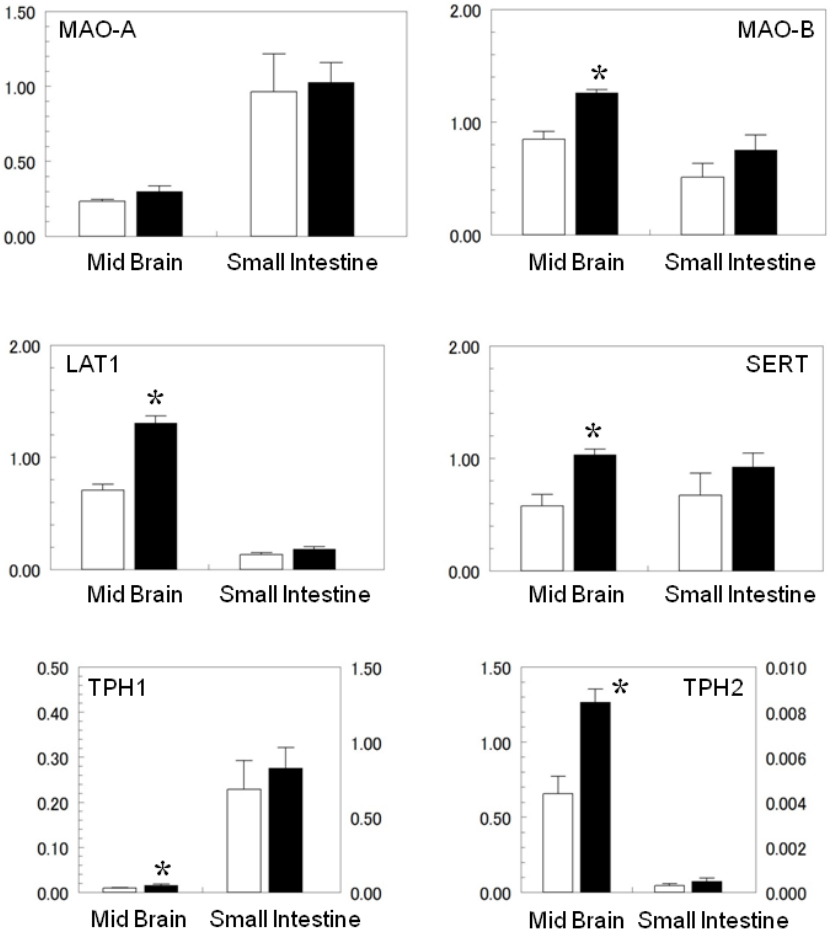

Figure 2. Serotonin-related mRNA levels in the midbrain and small intestine. Monoamine oxidase type B (MAO-B), serotonin transporter (SERT), tryptophan transporter (LAT1), and tryptophan hydroxylase type 2 (TPH2) mRNAs were significantly increased 6 hr after parabolic flight. The unit of measurement on the right axis in LAT1, SERT, MAO-A, and MAO-B is the same as that on the left axis. Black columns represent 6 hr after parabolic flight, and white columns represent just prior to the first parabola. Bars represent the means ± SEM of 5 animals per group. * p<0.05 vs. control.

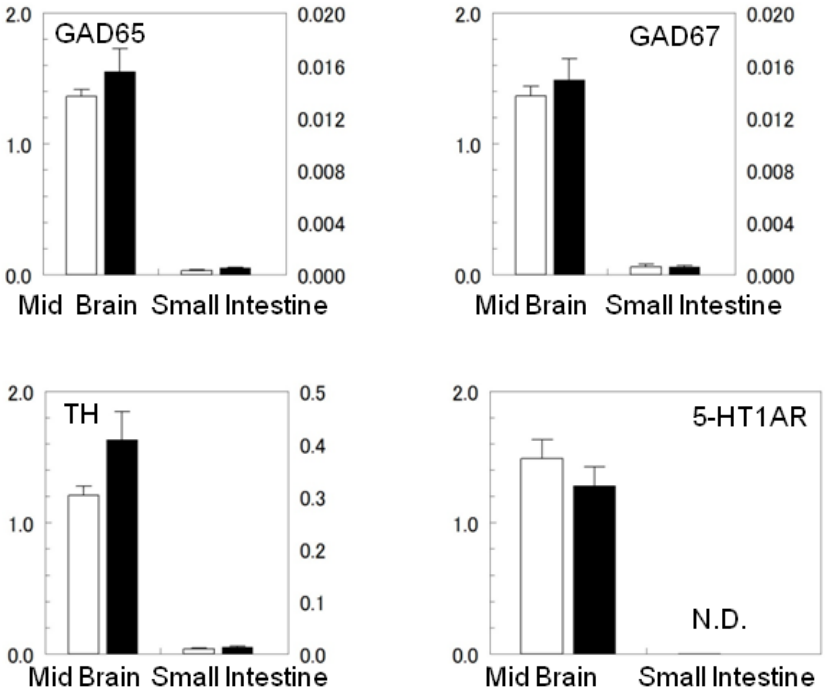

Figure 3. GAD, TH, and 5-HT1AR mRNA levels in the midbrain and small intestine. No significant changes in mRNA expression in glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH), and the serotonin 1A receptor (5-HT1AR) were observed following parabolic flight in the midbrain or small intestine. Black columns represent 6 hr after parabolic flight, and white columns represent prior to the first parabola. Bars represent the means ± SEM of 5 animals per group. N.D.=non-detectable.

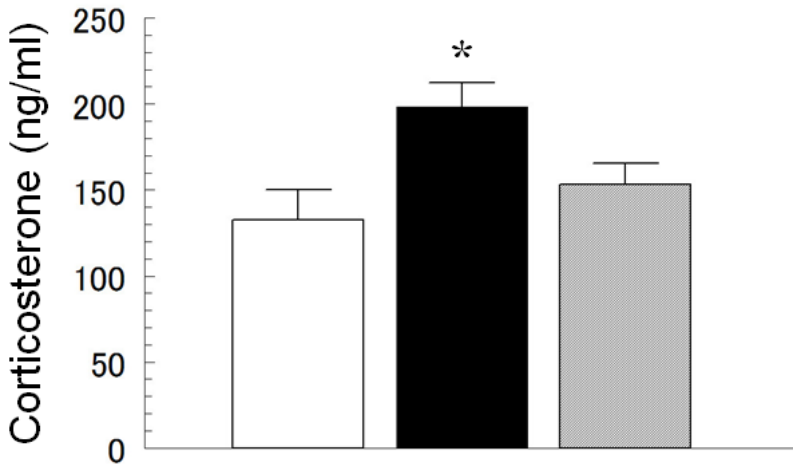

Figure 4. Changes in plasma corticosterone levels. Plasma was collected after decapitation at each point and assayed using a commercial radioimmunoassay (RIA) kit. Corticosterone levels were determined in duplicate in each study. The black column represents just after the last parabola, the white column represents prior to the first parabola, and the shaded column represents 6 hr after parabolic flight. Bars represent the means ± SEM for 7-8 animals per group. * p<0.05 vs. prior to the first parabola group.

DISCUSSION

Quantitative real-time PCR demonstrated that parabolic flight up-regulated SERT, LAT1, MAO-B, and TPH2 mRNA expression in the midbrain, including the raphe nuclei, but not 5-HT1AR, MAO-A, TPH1, TH, GAD65, or GAD67 mRNA.

TPH proteins are the rate-limiting enzymes in serotonin synthesis, and the LAT1 protein is a carrier for tryptophan, which is a TPH substrate. TPH2 mRNA is the predominant transcript in the raphe nuclei of mice (Walther et al., 2003a; Côté et al., 2003), rats (Patel et al., 2004), and humans (Zill et al., 2007), and TPH1 mRNA is predominantly expressed in the periphery, including the stomach and intestine (Walther et al., 2003b; Côté et al., 2003). The present study demonstrated that parabolic flights induced a 2-fold increase in TPH2 mRNA expression in the mouse midbrain, but TPH1 mRNA expression in the small intestine was not altered.

The mRNA expression of the serotonin synthetic enzyme and transport protein was increased by parabolic flight. Tryptophan, which is an essential amino acid and required substrate for serotonin biosynthesis, is transported by LAT1 because tryptophan cannot penetrate the phospholipid bilayer membrane. LAT1 is essential for the *de novo* synthesis of serotonin, and SERT contributes to the recycling of serotonin. Parabolic flight increased the mRNA of both transporters. The induction of THP2 and transporter mRNA in the brain suggests that an active system in the serotonergic neurons copes with unpredictable and novel events by the *de novo* synthesis and recycling of serotonin.

The serotonergic metabolic pathway was also affected by parabolic flight. The primary and initial step of serotonin metabolism is the oxidation that is catalyzed by MAO-A. Serotonin is predominantly oxidized by MAO-A, but other monoamines, including dopamine, are metabolized by MAO-B. Most serotonin-containing neurons in the raphe nuclei contain MAO-B but not MOA-A (Levitt et al., 1982). The presence of MAO-B in serotonin-containing neurons suggests a role for this enzyme in the regulation of other monoamine neurotransmitter levels in these CNS cells. A significant induction of MAO-B mRNA, but not MAO-A, suggested that serotonergic neurotransmitter was required for maximal and rational responses to dangerous and/or unpredictable negative situations.

GAD67 regulates basal levels of GABA, and GAD65 supplies GABA in sudden demand situations (Namchuk et al., 1997). GAD65 and

GAD67 mRNA were slightly but insignificantly up-regulated in the present experiment. These results suggest that the GABAergic system was initiated by parabolic flight.

Tyrosine hydroxylase (TH) is the key enzyme for dopamine and noradrenalin synthesis, and it also regulates the activity of both systems (Nestler et al., 1999). Exposure to stress during perinatal life alters norepinephrine noradrenaline biosynthesis, content, and turnover in the developing and adult rat brain (Matthews et al., 2001). TH mRNA was slightly but insignificantly up-regulated in the present experiment. This result suggests that the GABAergic was insensitive and not initiated by parabolic flight. The relationship between the catecholaminergic or GABAergic systems and parabolic flight requires further investigation.

Parabolic flight significantly increased serum corticosterone levels to 198.4 ± 14.0 ng/ml in mice (n=7). Our previous experiment in 10 human volunteers under the same parabolic conditions demonstrated that mean serum cortisol concentrations on the plane following parabolic flights reached 185.0 ± 25.8 ng/ml (Yoshioka et al., 2010). However, the basal cortisol levels prior to parabolic flight were different in mice and humans (132.9 ± 17.3 ng/ml and 76.0 ± 7.8 ng/ml, respectively). This difference may be due to a differential sensitivity for gravity changes, especially in weighted situations during ascent and prior to parabolic flight.

Glucocorticoid release during stress acts in the periphery and the brain to exert profound effects on endocrine function. These actions do not likely contribute to the immediate sympathetic fight-or-flight response that is necessary for survival in the face of an immediate threat. However, these actions are sufficiently rapid to sustain the short-term behavioral adaptations that are necessary for survival in a dangerous situation. A 4-day treatment with the synthetic glucocorticoid, dexamethasone, reduces TPH2 mRNA and protein in the raphe nuclei of mice (Clark et al., 2005), which suggests that stress hormones up-regulate TPH2 mRNA.

The results of the present study suggest that the serotonergic system in the CNS, particularly the synthetic pathway, was activated by gravity-changing stress. Further delineation of the anatomical and functional properties of specific stress- and anxiety-related serotonergic systems should aid our understanding of the neural mechanisms that underlie the etiology of anxiety and affective disorders.

In conclusion, the results of the present study suggest that the serotonergic system in the CNS, particularly the synthetic pathway, was activated by gravity-changing stress.

ACKNOWLEDGEMENTS

A special thank you goes to the team at Diamond Air Services for providing all of the support that we needed.

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