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Maladaptive Alterations of Defensive Response Following Developmental Complex Stress in Rats

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Objective: Despite the etiological significance of complex developmental trauma in adult personality disorders and treatment-resistant depression, neurobiological studies have been rare due to the lack of useful animal models. As a first step, we devised an animal model to investigate the effects of multiple trauma-like stress during different developmental periods.
Methods: Twenty-one male Sprague-Dawley rats were classified into 3 groups based on the stress protocol: fear conditioning control (FCC, n = 6), complex stress (ComS, n = 9), and control (n = 6). While the ComS experienced three types of stress (maternal separation, juvenile isolation, electric foot shock), the FCC only experienced an electric foot shock stress and the control never experienced any. We compared fear responses at postnatal day (PND) 29 and PND 56 through freezing time per episode (FTpE), total freezing time (TFT), total freezing episodes (TFE), and ultrasonic vocalization (USV).
Results: ComS showed the longest FTpE in the conditioned fear response test. ComS and FCC exhibited the longer TFT and these two groups only displayed USV. ComS show difference TFE between PND 29 and PND 56.
Conclusion: The results of this investigation show that complex stress may affect not quantity of fear response but characteristics of fear response. Longer FTpE may be associated with tonic immobility which could be considered as a failed self-protective reaction and might be analogous to a sign of inappropriate coping strategy and self-dysregulation in complex trauma patients.

KEY WORDS: Trauma; Animal model; Anxiety; Freezing reaction, cataleptic; Vocalization, animal.

INTRODUCTION

Human and animal studies have implicated stressful early-life experiences as a key risk factor for the development of psychiatric disorders [1-4]. The term complex trauma refers to multiple, chronic, repetitive experiences of traumatic events, most often in interpersonal nature and early life (e.g., sexual or physical abuse, neglect, war, community violence) [5,6]. Complex trauma affects victims throughout their entire life, and children exposed to complex trauma may show increased vulnerability to additional trauma and cumulative impairment [5,7]. Complex trauma also exhibits a strong association with the development of chronic treatment-resistant depression and borderline personality disorder in adulthood [8]. Despite its adverse effects, complex trauma in children is prevalent in the community [9-11].

For the development of effective intervention programs, studies have indicated that a better understanding and assessment of the mental health of patients with complex

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trauma are needed [9]. Patients with complex trauma are known to suffer from several psychiatric problems, including post-traumatic stress disorder (PTSD) and other comorbid disorders and functional impairments [5,12]. However, previous studies have only focused on specific, limited aspects of complex trauma, such as suicidality and early childhood regulatory and attachment disorders, and PTSD [13-15]. More recent studies have demonstrated that complex trauma affects the chronic self-regulation problems associated with maladaptive coping strategies and the capacity of self for altering one's behavior [5,16,17]. According to the description of complex PTSD in the International Classification of Disease, 11th version (ICD-11), complex trauma encompasses commonly prolonged or repetitive events from which escape is difficult or impossible (e.g., torture, slavery, genocide campaigns, prolonged domestic violence, repeated childhood sexual or physical abuse). Moreover, it is also characterized by severe and persistent 1) problems in affect regulation; 2) beliefs about oneself as diminished, defeated or worthless, accompanied by feelings of shame, guilt, or failure related to the traumatic event; and 3) difficulties in sustaining relationships and in feeling close to others [18]. Therefore, in order to understand the various aspects of complex trauma, it is necessary to understand self dysregulation in the patients of complex trauma.

Researchers have studied the immediate and long-term results of complex trauma, both biologically and behaviorally. While the neural basis of these abnormalities has not been fully elucidated, previous studies have suggested an association between childhood trauma and structural and functional abnormalities of brain regions mediating emotion, such as the hippocampus, amygdala, and prefrontal cortex [19-22]. However, there are limitations in population studies, because inducing trauma to humans has ethical issues and because it is difficult to carry out well-validated behavioral and psychophysiological studies in human populations. Therefore, previous studies of trauma surveying the neurobiological and genetic basis of anxiety and depression have used several animal models [23,24].

However, previous studies using animal models of complex trauma have some limitations. First, most animal models were based on both a single period and a single type of stress. Considering the life cycle, complex trauma in human life during perinatal, infancy, and juvenile period may correspond to that of rats in the prenatal, postnatal, and juvenile periods [25]. Maternal separation (MS) is a commonly used method to induce postnatal stress in rats [26,27]. Subjecting rats to MS can result in lasting changes in various measures of emotion-related behavior and stress-reactivity [28]. Other studies have focused on the long-term effects of several interventions on the prepubertal period (postnatal day [PND] 21 to PND 30−34) [29]. Juvenile isolation induces a variety of symptoms in rats, including depression-, anxiety-, and psychosis-like behaviors and signs of autonomic, neuroendocrine, and metabolic dysregulation [30-32]. Electric foot shock has also been incorporated as a stressor in various animal models of human disease, including anxiety, PTSD, and depression [33,34]. Second, although there are previous studies that have examined the effect of stress over different developmental stages [35,36], no previous studies have employed different stressors across different developmental periods, which is a hallmark feature in human complex trauma victims. In addition thereto, the results from currently used animal models are not uniform [37], and it also has been argued that each model of rats has a good ecological validity to model human mental illness [38]. Together, it will be helpful to employ multiple stressors across multiple development periods for building the animal model of complex trauma.

Because complex trauma makes patients vulnerable to additional victimization in other risky environments [16], we wanted to investigate how complex trauma maladaptively affects the defensive response in trauma-like environments. Typical behavior measurements in rat animal models (open field test, elevated plus maze and forced swimming test) are usually known to measure general and sustained symptoms, such as anxiety, unconditioned avoidance−approach behavior, and decreased locomotor activity in depression [39-41], rather than directly measuring behaviors related to the trauma-like situation. In contrast, fear conditioning reactions are defensive responses in the environment associated with stress [42], which can reflect vulnerability to further victimization, and a fear conditioning paradigm was developed to show the effects of chasing stress on sensitization to unconditioned stimuli [43]. Thus, by examining the effects of complex stress on the fear conditioning response, it may be possible to explore more directly the maladaptive alterations of complex stress in the defensive re-

sponse associated with further victimization.

Complex trauma is an important subject in psychiatry. Although an appropriate animal model is needed for the study of complex trauma, no animal model has been developed to demonstrate various traumas over several periods. In this study, we developed an animal model for studying complex trauma by applying different stressors at individual developmental stages. To examine the validity of the developed animal model, we examined anxiety-related behavioral characteristics.

METHODS

Animals and Housing

All experiments were conducted with offspring of three pregnant female Sprague-Dawley (SD) rats obtained from Orientbio Inc. (http://www.orient.co.kr/common/main.asp). Pregnant females at the first week of gestation period were individually transferred and given a 2-week habituation period. Ten to fifteen rats were born to each single parent. To exclude the effects of hormonal change, only male littermates were used in this study. The rats were housed in a climate-controlled laboratory environment (22 ± 1°C) under a 12 hours light/dark cycle (lights on at 7:00–19:00) with *ad libitum* access to food and water. On PND 2, male littermates from three parents were assigned to three groups, with male littermates from the same parent assigned to the same group: fear conditioning control (n = 6), complex stress (n = 9), or control (n = 6). The pups were weaned on PND 21 and housed under standard conditions, except the complex stress group (as described below). Stress protocols of each group are depicted in Figure 1A. The fear conditioning control group (FCC) experienced only electric foot shock stress at PND 28, and the control group never experienced any stress throughout the experiment. Both groups (FCC and control) were housed under standard conditions without any interventions during stress protocols. In the complex stress (ComS) group, all pups experienced three types of stress (maternal separation, juvenile isolation, electric foot shock). All animal procedures were performed in accordance with the US National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System (approval no. 2017-0003).

Separation Stress Protocol (Postnatal and Juvenile)

For conveying maternal separation (MS) in the neonatal period (PND 0–21), pups of the ComS group were removed from the home cage and placed inside incubators for 3 hours (10:00–13:00) per day during PND 2–14, while pups in the other groups were maintained together with their littermates. The duration and period of separation were based on MS protocols in other studies [44,45]. Incubators were pre-warmed and maintained at 30 ± 1°C using an adjustable heat mat during separation for avoiding hypothermia. For accentuating stress, separated pups were physically separated from one another during maternal separation. After 3 hours of separation, pups were handled individually during transfer and, when returned, placed into the corner opposite of the nest.

Male SD rats in the ComS group were housed either individually in the same cages under standard conditions (as described 2.1) for 1 week from PND 21 (weaning age corresponding to pre-adolescence) to induce stress related to social isolation in the juvenile period (juvenile isolation) [30]. After 1 week of isolation, nine male SD rats were re-housed together under conventional housing conditions, in which three juvenile rats were housed together until PND 42 and each rat had been housed in a single cage from PND 43. The protocol for juvenile isolation was based on other previous studies [30,46]. The separation stress protocol is depicted in Figure 1B.

Foot Shock Stress and Fear Conditioning Protocol

To induce an excessive traumatic experience, rats in the ComS and FCC groups were transferred to the testing room and underwent experiments from 17 o'clock in the prepubertal period (PND 28). The foot shock experiment was performed using a computerized fear-conditioning system (Panlab startle and fear conditioning system; Harvard Apparatus, Holliston, MA, USA). After 3 minutes of habituation, the animals were presented with inescapable tone-foot shock pairings (pure tone: 30 seconds, 55 dB; electric foot shock: 1.0 mA, 2 seconds) three times at 30-seconds intervals. During the last 1 minute after the foot shock phase, no stimulus was presented [47]. Over a total of 7 minutes, locomotor activity and vocalization were recorded by a camera mounted on the rectangular foot shock chamber (250 [width] × 250 [depth] × 250 [height] mm). After each individual test session, the apparatus was completely cleaned with 70% alcohol to elimi-

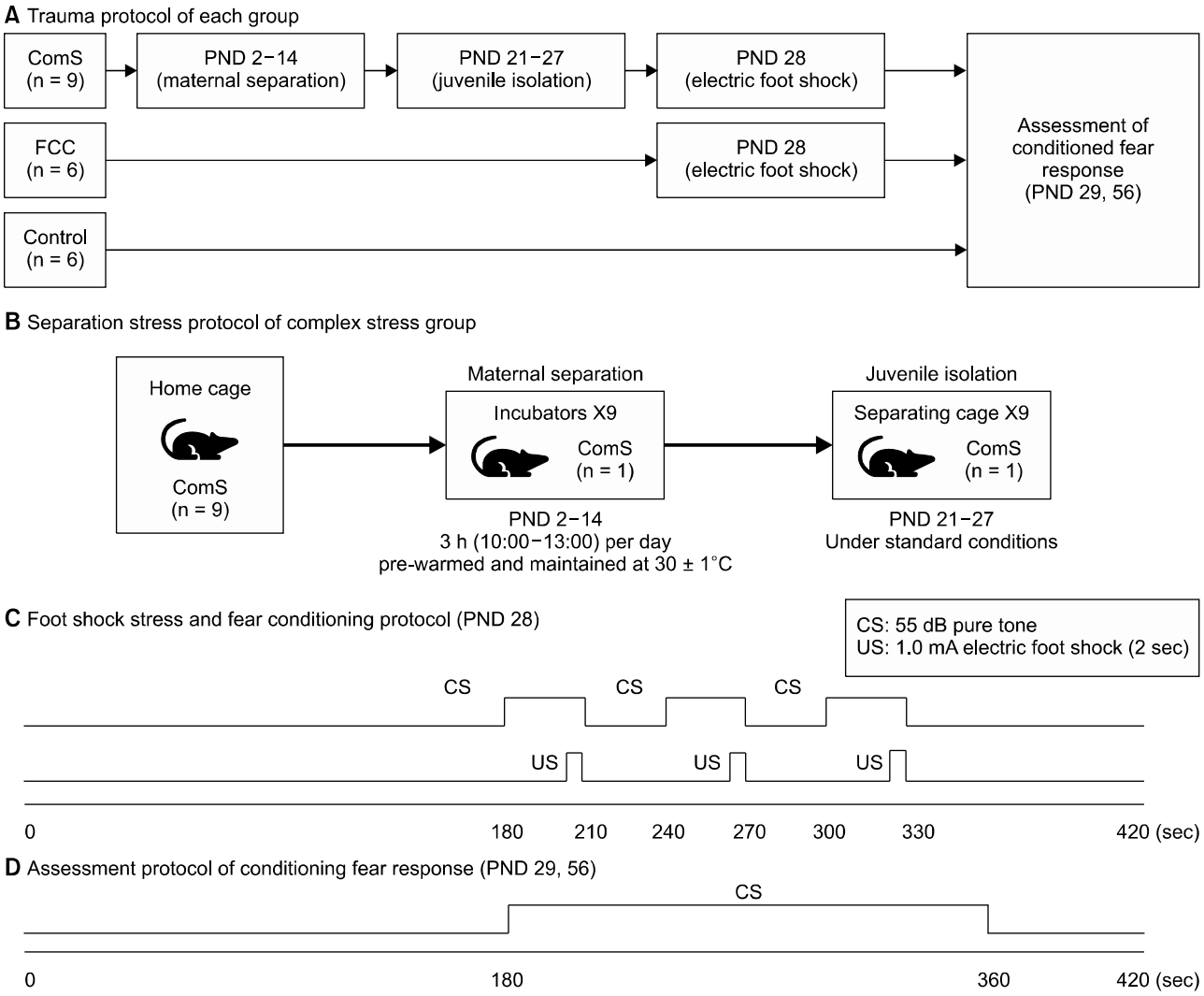

Fig. 1. Schematic representation of the protocol. (A) Overview of the stress protocols of each group. (B) Separation stress protocol of the Complex stress group. (C) Foot shock stress and fear conditioning protocol. (D) Assessment protocol of conditioned fear response.
ComS, complex stress; FCC, fear conditioning control; PND, postnatal day; CS, conditioned stimulus; US, unconditioned stimulus.

nate odor and defecation from the previous tested rats (Fig. 1C).

Assessment Protocol of Conditioned Fear Response

To examine the acute and lasting conditioned fear response, fear conditioning experiments were conducted at 1 day (PND 29) and 4 weeks (PND 56) after the foot shock stress [47]. All groups (including the control group) were assessed for conditioned fear responses in the same environment to the fear conditioning. This protocol differs from the previous fear conditioning protocol in that it only presents pure tone without paring electric shocks. Animals were presented with 3 minutes of pure tone and 1 minute without any auditory stimulus after a 3-minutes habituation period. After each test session, the apparatus was completely cleaned with 70% alcohol to eliminate odor and defecation from the previously tested rats (Fig. 1D).

Behavioral Data Collection

We collected data measured at PND 29 and PND 56 and used the Any-maze® (Stoelting Co., Wood Dale, IL USA) behavioral tracking software to detect a freezing response in the foot shock chamber. We recorded every test session using a camera mounted on the top of the chamber. The video files were transferred to the Any-maze® program, which automatically analyzed total freezing

time (TFT) and total freezing episode (TFE). Freezing was defined as a total absence of a body or head movement, except that associated with breathing [48]. A single "freezing episode" was defined by continuous freezing behavior over 2 seconds [49]. From TFT and TFE, freezing time per episode (FTpE) was calculated. Additionally, for measuring fear and anxiety related to conditioned fear responses, acoustical analysis of ultrasonic vocalization (USV) was used: we used the Petterson D-230 Bat Detector, which transforms high-frequency sounds (22 kHz) into the audible range [50]. The audio files were opened on Adobe Audition 3.0, and we filtered out any noise but USV. The total time of USV only during the 3-minutes tone in the conditioned fear response test was measured by an experienced user.

Statistical Analysis

The data are presented as means ± standard deviations of values following normal distribution and medians and ranges for those following non-normal distribution in Table 1. The data were analyzed using IBM SPSS Win version 23.0 (IBM Co., Armonk, NY, USA). For comparing the freezing behavior measurements of all groups that followed non-normal distribution, Kruskal−Wallis test and *post hoc* analysis including multiple pairwise comparisons adjusted by the Bonferroni procedure was used. For the USV time extracted from normally distributed variables, repeated measures of analysis of variance (ANOVA) with between subject factors (i.e., stress conditions [FCC, ComS]) and within subject factors (i.e., time [PND 29, 56]) was used for analysis, and the Bonferroni's method and paired t test were applied for *post hoc* test. Longitudinal comparison of freezing behavior measurements of each group was conducted by paired t test for values following normal distribution and by Wilcoxon signed rank test for values following non-normal distribution. A p value of 0.05 was set as the level of significance.

RESULTS

Freezing Time per Episode

FTpE, TFT, TFE, and USV at PND 29 and PND 56 are shown in Table 1. Statistical analysis revealed that FTpE was significantly different among groups at PND 29 ($p < 0.001$) and PND 56 ($p < 0.001$). In the *post hoc* test for FTpE, ComS rats showed the longest time per episode than any of the other groups at PND 29 (FCC: $p = 0.015$, control: $p < 0.001$). The ComS and FCC group rats froze longer than the controls at PND 56 (ComS: $p < 0.001$, FCC: $p = 0.027$); however, there was no significant difference in FTpE at PND 56 between ComS and FCC ($p = 0.339$). There was no significant difference in FTpE between PND 29 and PND 56 in all groups (ComS: $p = 0.066$, FCC: $p = 0.345$, control: $p = 0.593$) (Fig. 2).

Total Freezing Time

TFT at both PND 29 and PND 56 were significantly different among groups (PND 29: $p < 0.001$, PND 56: $p < 0.001$). In *post hoc* test, the ComS and FCC groups displayed longer TFT than controls at PND 29 (ComS: $p < 0.001$, FCC: $p = 0.002$) and PND 56 (ComS: $p < 0.001$, FCC: $p = 0.004$), although there was no difference in TFT between ComS and FCC rats in either PND 29 ($p = 0.816$) or PND 56 ($p = 0.339$). There was no significant differ-

Table 1. Freezing behavior and USV at PND 29 and PND 56

Variables	PND	Group		
		ComS (n = 9)	FCC (n = 6)	Control (n = 6)
FTpE	PND 29	59.80 (13.80−179.70)	13.72 (10.06−24.22)	0 (0−7.20)
	PND 56	17.05 (6.22−89.65)	9.72 (2.98−44.75)	0 (0−3.98)
TFT	PND 29	171.90 (41.40−179.70)	118.20 (80.50−147.80)	0 (0−7.20)
	PND 56	155.00 (80.80−179.30)	117.10 (17.90−179.00)	0 (0−19.90)
TFE	PND 29	3 (1−5)	9.00 (5−10)	0 (0−3)
	PND 56	9 (2−14)	11.00 (4−15)	0 (0−5)
USV	PND 29	20.52 ± 30.44	24.18 ± 26.43	
	PND 56	74.32 ± 47.34	101.88 ± 36.59	

Values are presented as mean ± standard deviation or median (interquartile range).
USV, ultrasonic vocalization (sec); PND, postnatal day; ComS, complex stress; FCC, fear conditioning control; FTpE, freezing time per episode (sec); TFT, total freezing time (sec); TFE, total freezing episode.

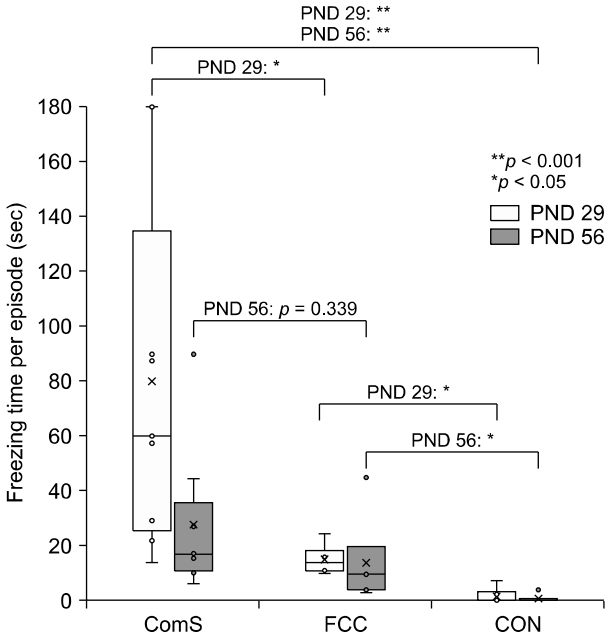

Fig. 2. The effect of each stress condition on freezing behavior in FTpE of each group. For each box, the central mark shows the median, the edges of the box represent the 25th and 75th percentiles, and the whiskers extend to the most extreme data points that are not considered outliers. The ends of the whiskers represent the lowest data value still within 1.5 times the interquartile range (IQR) of the lower quartile and the highest data value still within 1.5 times the IQR of the upper quartile. Kruskal−Wallis test. Exposure to the complex stress condition increased FTpE, compared to a single stress condition or no stress.
FTpE, freezing time per episode; ComS, complex stress; FCC, fear conditioning control; CON, control; PND, postnatal day.

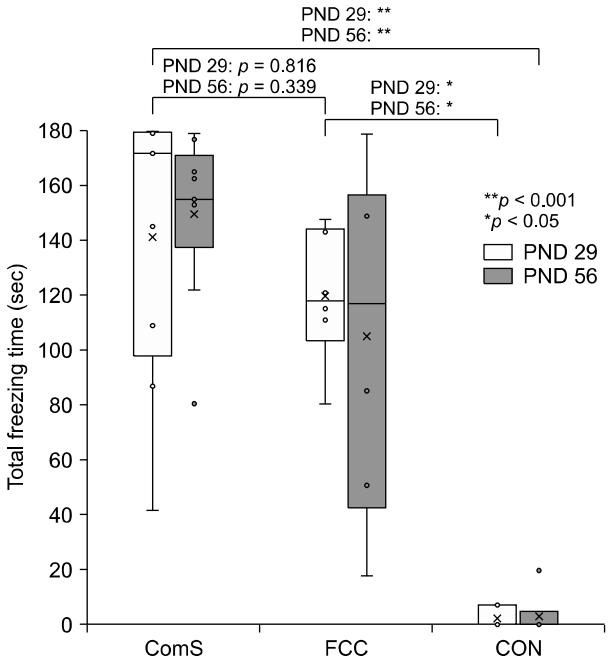

Fig. 3. The effect of each stress condition on freezing behavior in TFT for each group. For each box, the central mark shows the median, the edges of the box represent the 25th and 75th percentiles, and the whiskers extend to the most extreme data points that are not considered outliers. The ends of the whiskers represent the lowest data value still within 1.5 times the interquartile range (IQR) of the lower quartile and the highest data value still within 1.5 times the IQR of the upper quartile. Kruskal−Wallis test. ComS and FCC rats froze significantly more time than controls.
TFT, total freezing time; ComS, complex stress; FCC, fear conditioning control; CON, control; PND, postnatal day.

ence in TFT between PND 29 and PND 56 in all groups (ComS: $p = 0.678$, FCC: $p = 0.753$, control: $p = 1.000$) (Fig. 3).

Total Freezing Episode

There were significant differences in TFE at both PND 29 ($p = 0.001$) and PND 56 ($p = 0.004$). In the *post hoc* test, ComS and FCC rats show significantly increased TFE, compared to controls, at PND 29 (ComS: $p = 0.018$, FCC: $p = 0.002$) and PND 56 (ComS: $p = 0.002$, FCC: $p = 0.004$). There was a significant difference in TFE between ComS and FCC rats only at PND 29 ($p = 0.001$), not at PND 56 ($p = 1.000$). There was a significant difference in TFE between PND 29 and PND 56 only in the ComS group (ComS: $p = 0.013$, FCC: $p = 0.416$, control: $p = 1.000$) (Fig. 4).

Ultrasonic Vocalization

ComS and FCC groups of rats emitted spontaneous USV, while the control group did not. Repeated measure ANOVA showed the main effect of time ($F1 = 25.842$, $p < 0.001$); however, there were no significant interactions between group and time ($F1 = 0.841$, $p = 0.376$) and no group effects ($F4 = 1.161$, $p = 0.301$) (Fig. 5).

DISCUSSION

In this study, we attempted to show for the first time that complex stress exposure may have a differential effect on conditioned fear responses as an anxiety-related behavior in rats. ComS rats showed significantly longer FTpE than other rats. Because freezing episode was defined as freezing over 2 seconds without motion in this study, we considered that a long freezing time per single episode might be associated with tonic immobility (TI), which is a sus-

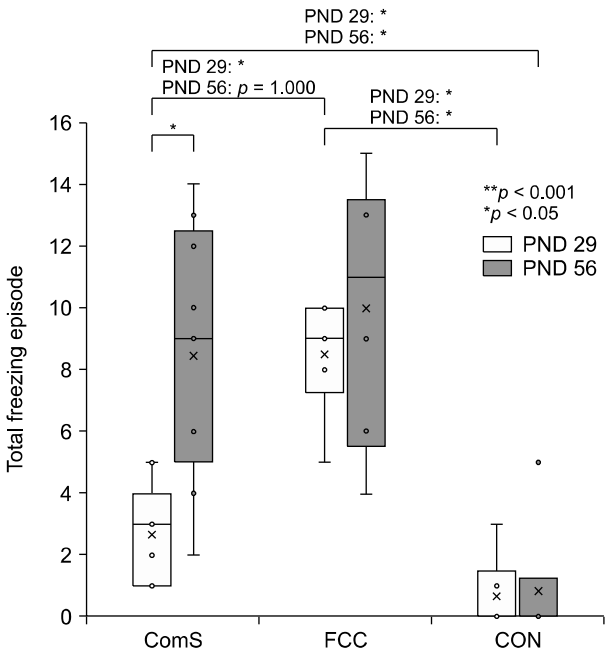

Fig. 4. The median TFE of ComS, FCC, and control groups. For each box, the central mark shows the median, the edges of the box represent the 25th and 75th percentiles, and the whiskers extend to the most extreme data points that are not considered outliers. The ends of the whiskers represent the lowest data value still within 1.5 times the interquartile range (IQR) of the lower quartile and the highest data value still within 1.5 times the IQR of the upper quartile. Kruskal–Wallis test. ComS only increased in TFE at PND 56, compared to PND 29.
TFE, total freezing episode; ComS, complex stress; FCC, fear conditioning control; CON, control; PND, postnatal day.

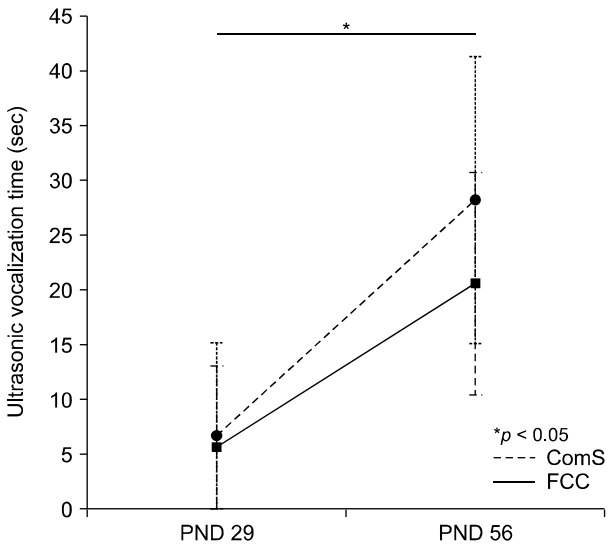

Fig. 5. Ultrasonic vocalization time for ComS and FCC rats at PND 29 and PND 56. Both groups displayed an increased ultrasonic vocalization time at PND 56, compared with PND 29.
ComS, complex stress; FCC, fear conditioning control; PND, postnatal day.

tained, profound, and reversible physical immobility. TI in humans is also presented as a sustained and largely involuntary pattern of neuromuscular activity (i.e., cataleptic-catatonic) [51,52]. If longer FTpE can be considered as an indicator of TI, this result may suggest that complex stress is associated with TI.

Compared to the control group, the ComS and FCC groups exhibited longer TFT, and only these two groups displayed USV. The behavioral profiles in these tests were consistent with previous studies demonstrating increased defensive responses in fear conditioning with tone and foot shock following traumatic stress treatment [47]. In the *post hoc* test, ComS rats did not show a significant difference in TFT and USV time, compared to FCC rats. One possible explanation for this result is a ceiling effect: TFT was skewed towards the upper bounding time of 180 seconds (Fig. 1) in both ComS and FCC rats. This result may also be explained by the difference in the effect of complex stress in characteristics of fear response. We can surmise that complex stress may not affect the total quantity of a fear response, but may affect the characteristic of fear responses. However, due to limitations of this study's design, their respective contributions cannot be disentangled.

Only ComS rats showed a difference in TFE between that at PND 29 and that at PND 56, and there was no difference in TFE at PND 56 between ComS and FCC rats. This result may imply that freezing behavior in ComS rats at PND 29 was qualitatively different from freezing behaviors in other groups and at other assessment times. Previous fear conditioning research showed that conditioned fear responses lasted more than 4 weeks after electric foot shock [47,53]. Therefore, it can be assumed that additional factors other than electric foot shock might affect ComS, showing a different TFE at PND 29. Because both ComS and FCC rats were in a frozen state most of the time, decreased TFE meant sustained, ongoing general physical immobility. Therefore, decreased TFE might be associated with TI, which is defined as ongoing general physical immobility [40]. These results may also be linked to previous work comparing dissociation in a clinical population who were sexually assaulted and TI in animals [54-56]. Because TI is especially associated with failure of integrating information and reacting appropriately and loss of chance to avoid further damage, TI can lead ComS group accident-proneness [57-59].

Additionally, the control group exhibited no USV. Because the control group did not experience any stress protocol, including electric foot shock as a negative control, this result supported the validity of the experiment. Unlike in FTpE of ComS (Fig. 2), USV time did not show a statistically significant difference between ComS and FCC groups, even though the ComS group showed increasing USV over time (Fig. 5). This finding may be interpreted as FTpE and USV reflecting different aspects of fear- and anxiety-related behaviors. The difference in incremental trends in USV and freezing behavior has also been shown in other studies of fear conditioning. In other studies, USV was more prevalent during inter-trial intervals of fear conditioning sessions than during the presence of the conditioned stimulus [55], and the amount of freezing reflected the strength of conditioned stimuli [60]. Suppressed vocal behavior was also described as a characteristic of TI in previous research [61,62]. It is likely that FTpE is related to more intense fear-related behavior than that of USV. However, as freezing time per episode was not studied widely as a feature of fear response, the interpretation of this result requires replication in future studies.

We did not directly observe other catatonic features in the freezing state, which is a limitation obscuring our interpretation of FTpE as being strongly associated with TI (i.e., hypertonicity, catatonic-like motionless posture, unresponsiveness to painful stimulation) [63,64]. It is necessary to involve conventional measurements for anxiety-related behavior (open field test, forced swimming test, and elevated plus maze) to understand the effects of complex trauma more comprehensively and to validate FTpE as a tool for evaluating anxiety in future study. Not investigating behavior in the fear conditioning experiment with rats that experienced only maternal separation and juvenile isolation is a limitation to interpreting the results of the current study. Because this study included only a small number of male rats, the question about possible sex differences in the outcome of complex stress could not be ascertained, and there is a limitation to generalizing interpretations of this study. Given the difference between female and male rats, further studies of the effects of hormonal changes in females are essential. Furthermore, evaluating objective measures in the animal model is also necessary to confirm the validity of the animal model in the current study. Therefore, further study should examine FTpE with fear response, including TI, more directly by investigating other features of fear responses with larger samples and with objective measures, including cortisol and epigenetic changes in experimental animals

This is the first study to investigate the possibility of differential fear responses in a complex stress animal model. We noted increased FTpE at PND 29, compared to PND 56, in only ComS rats, not in the others. We suggest that longer FTpE may be associated with TI, which may be considered as a sign of inappropriate coping strategies in complex trauma patients. In addition, because inappropriate coping strategies, such as dissociation, make patients vulnerable to additional victimization, we also suggest that increased FTpE and decreased TFE at PND 29 may hold important meaning in research of the effect of complex stress in animal models.

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■ Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

■ Author Contributions

Conceptualization: Junhyung Kim, June-Seek Choi, Chul Hoon Kim, and Jeong-Ho Seok. Data acquisition: Minkyung Park, Chiheon Lee, Jung Jin Ha, and Jeong-Ho Seok. Formal analysis: Junhyung Kim. Funding: Jeong-Ho Seok. Writing−original draft: Junhyung Kim, Minkyung Park, and Jeong-Ho Seok. Writing−review & editing: Junhyung Kim and Jeong-Ho Seok.

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