筑 波 大 学

博 士 （ 医 学 ） 学 位 論 文

The effect of acute social defeat stress on sleep in mice

（マウスにおける社会敗北ストレスの睡眠に対する即時的影響についての検討）

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Abbreviations

EEG   electroencephalogram

EMG   electromyogram

HPA   hypothalamic-pituitary-adrenal

REMS   rapid eye movement sleep

SoD   social defeat

SWA   slow wave activity

SWS   slow wave sleep

Background

Stress response and cost of allostasis

Homeostasis is the dynamic state of equilibrium of physiologic systems such as body temperature, oxygen tension, pH, blood glucose level and ion composition of extracellular fluid, which is essential for life and has to be maintained within a certain range by all living organisms. A stressor is a stimulus that challenges homeostasis, and animals respond with physiologic and behavioral changes in an attempt to maintain or restore homeostasis, which is called stress response (Chrousos, 2009). The psychological aspect of the stressor is known to be of great importance in stress response. Uncontrollability and unpredictability of aversive stimuli are generally associated with severe stress (de Boer et al., 1990; Ferrari et al., 2003; Koolhaas et al., 2011). Thus, stress is considered a state where homeostasis is actually threatened or perceived to be so. The stress response is considered to be aimed at adaptation to the changing natural environment for better survival and ultimately, for better reproduction of the individual in natural environment (Korte et al., 2005).

Stress response includes relatively stereotypic and innate reactions which were acquired through evolutional process (Chrousos, 2009). Central nervous system plays the

major role in regulation of the stress response. Arousal and vigilance are promoted in acute stress, and the excitations in hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system typically play major physiologic roles in stress response (Chrousos, 2009). Excitation of HPA axis increases the secretion of glucocorticoids, which exerts pleiotropic action throughout the body (Chrousos and Kino, 2005). The reactions prepare the animal to perform behavior required in urgent situations. For example, elevated levels of catecholamines and glucocorticoids promote energy mobilization through stimulation of glucogenesis, glycogenolysis, lipolysis and hepatic glucose secretion, and increased heart rate and arterial blood pressure by sympathetic activity promote distribution of the nutrients and oxygen to active organs such as skeletal muscles, the heart and the brain (Sapolsky et al., 2000). Moreover, shortly nonadaptive behavioral and physiologic functions, such as eating, growth and reproduction, are prevented (Chrousos, 1998, 2008). The glucocorticoids prevent inflammation, which may hamper behavioral performance, through suppression of proinflammatory cytokine secretions (Boumpas, 1993; Chrousos, 1995), although the effect of stress on immune system is rather complex, and increase of proinflammatory cytokines could also occur after acute stress presumably in preparation for possible injury during stress (Steptoe et al., 2007; Chrousos, 2009).

Although the stress response is ideally beneficial to handle the situation, it also costs animals (Chrousos, 2009). This is well explained in the concept of "allostasis" with clear terminology, while the word "stress" is often used in ambiguous ways (McEwen, 1998; McEwen and Wingfield, 2003). Allostasis is defined as the adaptive process where animals actively achieve stability through physiologic, behavioral and psychological changes, i.e., stress response described above in the context of adaptive process. The state with sustained activity of allostasis is called allostatic state. Animals may get into allostatic state in confrontation with social conflict, weather change, natural disasters, predator emergence, etc. Each animal is considered to have a certain capacity to cope with situations, and allostatic state imposes cumulative cost on the animal, which is called allostatic load. If the physiologic and psychological capacity of an individual is put in danger by the intensity and/or frequency of stressor, allostatic load can expand intensely, leading to allostatic overload. In such case, the individual's health is in danger. The shortly adaptive alterations in allostasis start to damage itself. For example, prolonged stress increases the risk of cardiovascular diseases such as hypertension, atherosclerosis and coronary heart disease (Melin et al., 1999; Kaplan et al., 2009; Khayyam-Nekouei et al., 2013; Rozanski, 2016) and gastric ulcer formation (Weiner, 1996; Grundy et al., 2006; Deding et al., 2016). In the hippocampus, which is important in spatial and episodic

memory and mood regulation, dendritic shrinkage and loss of spines are induced by stress, where glucocorticoids play a major role (McEwen, 1999; Popoli et al., 2012; McEwen et al., 2015). Disruptions also occur in immune system or energy metabolism by glucocorticoids, catecholamines and other mediators (Glaser and Kiecolt-Glaser, 2005; Tamashiro et al., 2011; Hirotsu et al., 2015). Furthermore, the behavior may be altered in maladaptive ways, causing psychological disorders, e.g., depression and post-traumatic stress disorder (Charney and Manji, 2004; Huhman, 2006). Uncontrollability and unpredictability of stressors, to which animals have difficulty in adjusting their behavior, are considered especially important for the development of stress-related disorders (Koolhaas et al., 2011).

It is known that coping styles to stressor are different among individuals even in the same species (Koolhaas et al., 1999, 2007; Korte et al., 2005). Differences are observed not only in behavior but also in physiologic stress response. A certain coping style may be beneficial in certain conditions and can be also disadvantageous in other conditions. In feral populations of rodents, for example, their coping styles were characterized between proactive and reactive coping (Koolhaas et al., 1999). Some male animals tend to show strong intermale aggression, whereas others are not aggressive. The individual difference of aggression level is known to be related to the general coping

styles in which they react to various environmental challenges. Aggressive animals usually show proactive coping. Proactive animals easily develop routines, in which the behavior is rigid and stereotypic, responding relatively independently of the environmental stimuli. On the other hand, reactive animals, which are not aggressive, are more flexible in their behavior and sensitive to environmental changes. The difference in their coping styles has been studied in various laboratory settings (Van Oortmerssen et al., 1990; Benus et al., 1991; Koolhaas et al., 1999). For example, in the learning task in a maze to get food as reward in mice, when the position of food was changed in the maze, it was revealed that reactive mice were more flexible in changing their behavior than proactive mice. Contrary, in response to a small change in the maze, such as a small piece of tape in the floor or angle change of the maze relative to outside cues, it is reported that proactive mice did not pay attention to the change, and the performance was not influenced, whereas reactive mice showed exploration on the maze again and took much more time and errors on the task (Van Oortmerssen et al., 1990; Coppens et al., 2010). Thus, a proactive coping style may be beneficial in stable environmental conditions, in which quick behavior based on previous experience is effective. The reactive coping animals may perform better in unstable changing environmental conditions because they are more aware of changes. In addition to the behavioral difference, is also reported that

proactive or reactive animals show higher responsivity to stressors in sympathetic activity

or HPA axis respectively (Koolhaas et al., 1999). Importantly, the variation in coping style

is considered to have been developed through natural selection, in which higher

performance in reproduction is considered more successful. Thus, the individual's health

is not necessarily the goal of the stress system (Korte et al., 2005).

Then, how is sleep-wake behavior influenced by stress and involved in the

adaptation process?

Social defeat stress models

The effect of stress has been studied in various animal models, including

restraint/immobilization stress, electric shock, ether exposure and social defeat (SoD)

(Sutanto and de Kloet, 1994). The acute physiologic reactions such as HPA axis and

sympathetic activation as well as adverse consequences by sever or chronic stress have

been studied (Koolhaas et al., 1997, 2011; Willner, 2005; Nestler and Hyman, 2010).

Among these stressors, social conflicts often occur in natural contexts in social animals,

and SoD stress has great impacts on their behavior and physiology. SoD stress has been

used as ethologically relevant models of stress (Toyoda, 2017).

Social animals such as rodents live in social rank, which is usually decided by

winning and losing in social conflicts. The rank is a critical determinant of resource

allocation like territory, food, shelter and chance of reproduction. Thus, wining in a

conflict can be of great importance. Persistent fighting of an individual with difficulty to

dominate its opponent, however, may not be adaptive because injury and death could

happen. In addition, it is also suggested that the dominant animals with difficulty in

maintaining their social rank have an increased risk in a series of cardiovascular disorders,

whereas subordinate animals usually do not (Ely, 1981; Koolhaas et al., 1999; Kaplan et

al., 2009). To avoid unpreferable consequences, animals show submissive behavior and

accept a lower social rank in which available resources are compromised. In humans,

social conflicts are a major source of stress, and exposure to SoD such as loss in

aggressive social confrontation, being bullied or being abused, is associated with

increased risk in depression, anxiety and post-traumatic stress disorder (Agid et al., 2000;

Björkqvist, 2001; Heim and Nemeroff, 2001; Charney and Manji, 2004). Due to this

interest, the effect of social conflict of animals has been studied intensively in the losers.

Rodent studies of SoD stress are often conducted in a resident-intruder paradigm.

The paradigm is based on the aggressive behavior of a resident animal toward an

unfamiliar male intruding in its home cage. It is known that territorial aggression of adult

male rats and mice is strongly enhanced in the presence of females and/or sexual

experience (Goyens and Noirot, 1975; de Catanzaro, 1981; Albert et al., 1988). Thus, resident animals are generally prepared from breeding colonies and/or kept with females (Golden et al., 2011; Koolhaas et al., 2013). In the paradigm, male intruder animals are put in a male resident's cage, and eventually subordinate themselves to the territorial resident conspecific with submissive behaviors such as submissive posture, escaping or freezing behavior when attacked and defeated. After the physical SoD, the intruder is often subjected to different durations of psychological stress of olfactory, auditory and visual stimulus through wire mesh or perforated partition which is used to prevent further physical contact. The effects of SoD have been studied in the intruder animals. Acute SoD stress stimulates sympathetic system and HPA axis and increase the heart rate, the body temperature and the blood corticosterone level (Koolhaas et al., 1997; Kataoka et al., 2014). These stress responses dissipate and return to baseline levels within hours after the end of the stress period. SoD stress, however, also induces longer-lasting behavioral and physiologic changes. In rats, for example, locomotor activity in the open field test was decreased for one week or more, and the circadian amplitude of the body temperature was decreased for several days mainly due to increased body temperature during the circadian rest phase after a single SoD (Meerlo et al., 1996a, 1996b). Furthermore, body mass growth was decreased for several days. When SoD was repeated for 2 consecutive days,

the decrease in the growth became much stronger with decline of food intake (Meerlo et al., 1996c). These observations indicate that SoD is a big life event in social animals, and even a single SoD can have a great impact on their physiology and behavior. Moreover, social animals are known to develop depression-like behavior in response to chronically repeated SoD stress (Von Frijtag et al., 2000; van Kampen et al., 2002; Rygula et al., 2005; Huhman, 2006). In mice, the procedure of chronic SoD stress has been well established, in which a male C57BL6j mouse (intruder) is socially defeated by an unfamiliar male CD-1 mouse (resident) with larger body size in daily basis for 10 days. Each day, the intruder mice are subjected to psychological sensory stress through perforated partition all day after 5-10 min of physical interaction with a CD-1 resident. After the chronic SoD stress, mice show depression-like behavior including anhedonia, anxiety, and social avoidance (Golden et al., 2011; Henriques-Alves and Queiroz, 2015). The anxiety and social avoidance behaviors can last at least 4 weeks (Berton et al., 2006; Tsankova et al., 2006; Krishnan et al., 2007). These long-lasting behavioral changes in animals that experience repeated SoD stress are commonly associated with alterations in gene expression patterns in the brain (Tsankova et al., 2007).

Function and regulation of sleep

Although stress coping of animals and humans is usually explained based on the state of wakefulness, the brain works with three distinct vigilance states, which are wakefulness, slow wave sleep (SWS) and rapid eye movement sleep (REMS) in mammals. Those states are relatively clearly defined primarily based on the electroencephalogram (EEG) signal. Sleep or sleep-like state is observed not only across animals but also in invertebrate such as drosophila (Hendricks et al., 2000; Shaw et al., 2000). Sleep is considered to be a critical process in which the brain actively maintains its functions, which has long been possessed throughout the evolutional process even with the fact that animals need to stay defenseless and cannot work to gain materials for living during sleep (Rial et al., 2010).

Sleep has been reported to have numerous functions, which may help animals to adjust their physiology and behavior on stress exposure. One of the most well-known function is its role in memory. Sleep has critical roles in memory consolidation. Memory consolidation is a process where initially unstable memories created during the awake state are transformed into more stable representations, which involves the integration into the network of pre-existing long-term memories. In human, for example, brief sleep (3h) after learning made emotional memory still detectable even after 4 years (Wagner et al.,

2006). It is well-known that neuronal re-activation occurs during SWS after learning tasks or exploration of a novel environment. The re-activation is observed in a similar spatio-temporal patterns of neuronal firing that occur in brain areas including hippocampus and cerebral cortices (Wilson and McNaughton, 1994; Ji and Wilson, 2007). The re-activation is suggested to be important in the memory consolidation during sleep (Rasch et al., 2007; Girardeau et al., 2009; Bendor and Wilson, 2012). REMS is considered to be important in consolidation or processing of emotional memory (Wagner et al., 2001; Nishida et al., 2009; Goldstein and Walker, 2014). Recently, the theta rhythm during REMS has been shown to be causally related to the contextual memory consolidation (Boyce et al., 2016). The differential functions of SWS and REMS in memory consolidation, however, is still obscure, and both types of sleep are important in normal memory consolidation (Diekelmann and Born, 2010). It is also reported as a structural evidence that dendritic spine formation in the motor cortex after learning tasks is promoted by sleep (Yang et al., 2014).

In addition to the memory function, sleep has a myriad of physiologic functions. For example, sleep enhances clearance of the metabolites such as beta-amyloid, which deposits in the brain tissue during awake period (Kang et al., 2009; Xie et al., 2013). It has also been hypothesized that sleep plays a role in the regulation of synaptic

homeostasis to maintain appropriate cognitive performance by downscaling synaptic strength to the baseline level (Tononi and Cirelli, 2003, 2006). Moreover, sleep also influence the endocrine system. Secretions of growth hormone or glucocorticoids are promoted or inhibited respectively (Takahashi et al., 1968; Weitzman et al., 1983). The immune system and the metabolic system of bodily energy are functioning in close association with sleep-wake cycle, where adequate sleep is critical in optimal functioning (Besedovsky et al., 2012; Morselli et al., 2012). Overall, sleep generally functions to maintain the optimal or regular physiologic state, which is contrastive to the allostatic state with homeostatic deviation during stress.

Animals need to adjust sleep/wake behavior to cope with changing environment. Although the vigilance states involve a brain-wide operational change, the switching and the propensity of those states are regulated by specific neural circuits, which integrate the internal states of the body and the brain as well as the external environmental condition through cognitive processes (Saper et al., 2005, 2010; Weber and Dan, 2016). Circadian rhythm and homeostatic regulation of sleep are the major modulators of the sleep/wake cycle (Borbély et al., 2016). The suprachiasmatic nucleus located in the hypothalamus serves the central pacemaker of circadian rhythm (Wurts and Edgar, 2000; Hastings et al., 2018). The suprachiasmatic nucleus is considered to play the regulatory effect on

sleep/wake behavior primarily through dorsomedial hypothalamic nucleus, which

innervates brain areas promoting sleep or wakefulness (Chou et al., 2002, 2003). In

addition to the direct neuronal regulation of sleep/wake cycle, because diverse

physiologic systems work along the circadian rhythm, sleep/wake behavior may also be

modulated indirectly, for example, through hormonal or metabolic system (Gnocchi and

Bruscalupi, 2017; Hastings et al., 2018). Homeostatic regulation of sleep is based on the

fact that after a prolonged wakefulness, animals normally sleep longer with higher

intensity (Borbély et al., 2016). High sleep intensity is expressed by enhanced slow wave

activity (SWA) of SWS-EEG, which gradually dissipates during the recovery sleep period

(Suzuki et al., 2013; Dispersyn et al., 2017; Wang et al., 2018). Homeostatic sleep need

is considered to accumulate during wakefulness. The primary mechanism of the sleep

need, however, remains still obscure in mammals. The endogenous sleep promoting

substances, such as adenosine, prostaglandin D_2, interleukin-1 and tumor necrosis factor-

α, have been discovered (Feldberg and Sherwood, 1954; Huang et al., 2011; Krueger et

al., 2011; Urade and Hayaishi, 2011). Among them, adenosine has been believed to play

important roles in homeostatic sleep regulation. Adenosine accumulates during

wakefulness in some brain areas such as cholinergic basal forebrain, hippocampus and

cortex (Huston et al., 1996; Porkka-Heiskanen et al., 1997, 2000). The adenosine exerts

its somnogenic effect through adenosine A_1 receptors and adenosine A_{2A} receptors in the central nervous system (Bjorness et al., 2009, 2016; Halassa et al., 2009; Huang et al., 2011). On the other hand, recently, the forward-genetics approach revealed that a dominant mutation in the gene of SIK3, which is a serine-threonine protein kinase, causes constitutively high sleep need (Funato et al., 2016). Further phosphoproteomic analyses indicated that mostly synaptic subset of neuronal proteins in the brain of the mutant mice are hyperphosphorylated (Wang et al., 2018). Importantly, the hyperphosphorylated state is also observed in the sleep-deprived mice, and the SIK inhibitor reduced elevated sleep need. Thus, this mechanism may be the central of cellular and molecular regulation of homeostatic sleep need although the mechanism linking the hyperphosphorylated state and the resultant expression of sleep need remains unknown. In addition to circadian and homeostatic sleep regulation, motivational processes also modulate propensity of sleep/wake behavior. For example, humans can stay awake late at night exceeding the normal time to sleep when motivated to work on cognitive or physical activities. Dopaminergic neurons arising from ventral tegmental area in the midbrain is considered to play an important role in the motivational regulation of sleep (Qu et al., 2010; Eban-Rothschild et al., 2016; Oishi et al., 2017a, 2017c).

Interaction between stress and sleep

To cope with external challenges, animals require alertness, and stress is a series of behavioral and physiologic responses to the challenge. Thus, stress commonly promotes wakefulness and inhibits or disrupts sleep. In fact, we often experience difficulties in falling or staying asleep at night when we are exposed to stressful life events, while the insomnia may be mitigated and resolved when individuals overcome or adjust themselves to stressful situations (Basta et al., 2007; Suchecki et al., 2009; Hirotsu et al., 2015). The transient insomnia may be adaptive for wild animals to survive with the prediction of external threat such as predator's attack that could occur during the normal sleep period. In humans, however, insomnia is generally undesirable and is one of the most common sleep problems. Chronic insomnia can be triggered by stressful life events in vulnerable individuals and is considered to be the state of hyperarousal, which is led by constant emotional arousal often with "fear of sleeplessness" (Basta et al., 2007; Kalmbach et al., 2018). Insomnia often occur simultaneously with the psychiatric disorders such as anxiety and depression, and disrupted sleep, in turn, contributes to the development and deterioration of the psychiatric disorders (Basta et al., 2007; Meerlo et al., 2008; Medina et al., 2014; Kalmbach et al., 2018; Steiger and Pawlowski, 2019).

On the other hand, after the removal from an acute stress exposure in animal

studies, alterations in the sleep architecture are often observed (Suchecki et al., 2009, 2012; Sanford et al., 2015). A sleep rebound may be induced in animals to compensate for the sleep loss during stressful situations, but it is believed that sleep alterations are not only a homeostatic response to the sleep loss but also created by the stress. Some stressors appear to induce even more sleep than the sleep actually lost during stress. The type of sleep that is induced and the extent to which sleep stages are enhanced are highly variable between the types of stress or even the study design. For example, acute immobilization or restraint for 1-2 h is followed by a selective increase in REMS (Rampin et al., 1991; Meerlo et al., 2001b), whereas inescapable footshock stress or learned helpless paradigm may not cause even a compensatory rebound of REMS (Adrien et al., 1991; Sanford et al., 2003a, 2003b, 2003c, 2010). In addition to the physical properties of the stressors, the controllability of the stressor also influences the following sleep responses. For example, an experiment was conducted on yoked pairs of mice experiencing the same amounts of footshock. One of the yoked pairs experienced escapable foodshock, where the mice can terminate the footshock by moving to the safe side of the experimental box, whereas the other experiencing inescapable foodshock cannot stop it by its action. Mice that experienced escapable footshock stress showed an increase in REMS, whereas mice with inescapable footshock stress showed a decrease in REMS (Sanford et al., 2010). On the

other hand, strong effect is observed on SWS after acute SoD stress. It is consistently reported that acute SoD stress increases SWA in the following sleep in rodents (Meerlo et al., 1997, 2001a; Meerlo and Turek, 2001; Kamphuis et al., 2015; Henderson et al., 2017). This suggests that SoD stress strongly enhances homeostatic sleep need. Moreover, one study reported that acute SoD stress in mice strongly increased the amount of SWS up to the 12 h period (Meerlo and Turek, 2001). The SWA enhancement was dissipated over the 6h period and SWA became even significantly less during the 12-18 period compared to baseline. This indicates that in this study, the extent of SWS increase in amount was much larger than needed to compensate the homeostatic sleep need that was imposed during SoD. Overall, stress can strongly enhance sleep depending on the stress protocols. Stress costs animals allostatic load. Sleep may function as a recovery process of the deviated homeostasis through its myriad of physiologic effects. The memory consolidation by sleep may help animals handle next challenges in better ways by optimizing their behavior for better survival in the natural environments.

Sleep deprivation is often used to experimentally enhance sleep pressure, which is observed in the following period as enhancement of SWA during SWS and/or increase of the amount of both types of sleep. Using sleep deprivation, investigation has been conducted to elucidate the molecular and neuronal mechanisms of accumulation of

homeostatic sleep need and sleep promotion after a prolonged wake period, and some

relevant mechanisms have been identified (Vyazovskiy et al., 2009; Huang et al., 2011;

Zhang et al., 2015; Gent et al., 2018; Wang et al., 2018). Adenosine has been considered

to be important in the recovery sleep, and phosphorylation of a series of neuronal proteins

in the brain is suggested to be the central molecular mechanism of the accumulation of

the homeostatic sleep need. On the other hand, experience during wake period is known

to affects the following sleep, and some stress protocols have strong effects (Suchecki et

al., 2009, 2012; Sanford et al., 2015). Especially, SoD stress has been reported to enhance

the intensity and/or the amount of the following SWS in rodents (Meerlo et al., 1997,

2001a; Kamphuis et al., 2015; Henderson et al., 2017). However, the mechanism of SWS

enhancement by SoD stress has not been investigated, and thus it is unknown whether the

mechanism of SWS enhancement is largely similar to that by the sleep deprivation or

involves other specific mechanisms. In humans, insomnia is often triggered by stress even

though stress itself may create the sleep need, and the disrupted sleep contributes to the

exacerbation of psychiatric disorders. The investigation of the sleep homeostasis in the

brain during and after stress may help understand the interrelation of sleep disruption and

psychiatric disorders.

Differences of the SoD effects on sleep among studies in mice

Although SWA is consistently enhanced during SWS after SoD stress in mice and rats (Meerlo et al., 1997, 2001a; Meerlo and Turek, 2001; Kamphuis et al., 2015; Henderson et al., 2017), the extent of SWS promotion in amount varies between SoD studies and protocols. Male C57BL/6j mice when defeated by aggressive mice of the same strain during a 1-h interaction period showed a strong increase of SWS (Meerlo and Turek, 2001), whereas only a small increase of the SWS amount was observed in another study using the same SoD procedure (Vaanholt et al., 2003). This difference may be explained by variations in the aggressive behavior of the resident mice or prior stress experiences of the intruder mice in the laboratory environment. When highly aggressive male CD-1 mice were used in another study for the SoD of C57BL/6j mice during a 5-min interaction period followed by a 20-min period of olfactory, visual and auditory contact between the resident and intruder mice, a SWS increase in the intruder mice was preceded by an increase of wakefulness (Henderson et al., 2017).

In the previous studies about effects of SoD stress on sleep in mice, one of the biggest concerns in interpreting the results is behavioral variations of the aggressive interaction. The intensity and frequency of attacks can vary in studies depending on the levels of aggressiveness of the residents used and the difference in body sizes between

residents and intruders. For example, in the SoD protocol with 1-h interaction with a mouse of the same strain described above, aggressive interactions and attacks may occur continuously or intermittently with periods of non-social interaction. The frequency of the aggression may influence the quality of the stress and lead to different results on the subsequent sleep. On the other hand, male CD-1 mice are often used to defeat C57BL/6j mice. Due to the difference of the strain, it is easy to prepare substantially bigger CD-1 mice relative to experimental C57BL/6j mice. In the protocol, the CD-1 mice are screened by a high level of aggression before use. The duration of physical interaction is usually set to 5-10 min. In this protocol, however, it is generally very difficult or impossible to prevent wounding of C57BL/6j mice during 5-10 min physical interaction period (Golden et al., 2011; Toyoda, 2017). A fixed interaction period likely leads to painful attacks of the CD-1 mouse on the intruder mouse and thus, the sleep-wake behavior of the intruder mice may also be affected by pain. It is also reported that wounding is not a required component in the outcome of chronic SoD stress measured by social avoidance (Krishnan et al., 2007). Thus, psychological nature is considered a significant characteristic of the SoD stress although physical contact is necessary for strong SoD. Overall, the extent to which the SoD procedure (e.g. SoD, pain or sleep deprivation) contributes to the post-SoD stress sleep/wake effects is not well established.

Purpose of the study

The purpose of the study is to establish post-SoD stress sleep/wake effects in a novel mouse model in which physical contact was controlled to minimize the behavioral variation during SoD. I developed a mouse model of SoD stress based on a resident-intruder paradigm to evoke sleep alterations in the intruder C57BL/6j mouse after acute SoD stress by the resident CD-1 mouse trained to display persistent aggression against the intruder mouse. In order to minimize behavioral variations and painful attacks to the submissive intruder mouse, the physical contact was interrupted when the intruder was attacked and showed submissive behavior instead of setting a fixed duration of a physical interaction. After the interruption, the intruder was separated by a partition with wire-mesh opening for psychological stress from the resident mouse. In order to maintain a high level of SoD stress in the intruders during the entire 1-h of SoD session, the partition was removed for physical contacts several times in a session. The effect of SoD stress on subsequent sleep was evaluated. By comparisons with several control procedures, the specific effect of SoD stress was investigated.

Materials and methods

Animals

Male C57BL/6j mice (13-20 weeks old, and weighing 26-33 g), maintained at the International Institute of Integrative Sleep Medicine of the University of Tsukuba, were used in the experiments. Male CD-1 (retired breeders) mice were obtained from Japan SLC (Hamamatsu, Japan). The animals were housed in an insulated and soundproof recording chamber maintained at an ambient temperature of 23 ± 0.5°C with a relative humidity of 50 ± 5% and an automatically controlled 12 h light/12 h dark cycle (illumination intensity ≈ 100 lux). All animals had free access to food and water. All procedures were in compliance with relevant Japanese and institutional laws and guidelines, and approved by the Institutional Animal Care and Use Committee of the University of Tsukuba.

Stereotaxic surgery for placement of the EEG/EMG electrodes

Mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneal [i.p.]) and then placed in a stereotaxic apparatus. EEG and electromyogram (EMG) electrodes for polysomnographic recordings were chronically implanted in the mice (Oishi et al., 2016).

The implant comprised two stainless steel screws (1 mm in diameter) that served as the

EEG electrodes inserted through the skull above the cortex (anteroposterior, +1.0 mm;

left-right, -1.5 mm from bregma or lambda) according to the atlas of Paxinos and Franklin

(2004). Two insulated, stainless steel Teflon-coated wires, serving as the EMG electrodes,

were placed bilaterally into both trapezius muscles. All electrodes were attached to a

micro connector and fixed to the skull with dental cement.

Stress procedures

The SoD stress protocol was designed based on the resident-intruder paradigm.

To prepare aggressive resident mice, male CD-1 (retired breeders) mice (body weight >

40 g) were singly housed upon arrival, and trained to display persistent aggression against

male C57BL/6j mice by placing a male C57BL/6j mouse in the cage of a CD-1 mouse

one or two times a week. During the training, the C57BL/6j mouse was immediately

removed from the cage when attacked and defeated by the CD-1 mouse. Unsuitable CD-

1 mice that showed little aggression or extreme violent behavior (i.e. mice that inflict

serious injuries to their opponent) were excluded from the study. Only CD-1 mice

showing aggression against C57BL/6j mice within 1 min were used in the experiments to

ensure successful SoD. The C57BL/6j mice used for aggression training were not used

for any behavioral experiments.

For SoD stress, I used a transparent acrylic partition placed diagonally in a rectangular cage to separate the intruder and resident mice. The partition has a wire-mesh opening in the lower part for olfactory, visual, and auditory contact (**Figures 1B,C**). A SoD session lasted 60 min starting at zeitgeber time 11. The C57BL/6j mouse (intruder mouse) was first placed behind the partition in the resident mouse's cage. After 5 min, the partition was removed for physical contact with the resident mouse. The partition was re-inserted when the intruder mouse was attacked by the resident mouse and showed clear submissive behavior, including submissive posture, escaping or freezing behavior. During one SoD session, the removal of the partition was repeated two, four or eight times at 25-min, 15-min or 7-min intervals, respectively. After the SoD session, the intruder mouse was returned to its home cage (**Figure 1D**).

The following experiments were also conducted to differentiate the specific effects of SoD and non-specific effects of the SoD procedure (**Figure 1E**): (1) C57BL/6j mice were placed in an unused cage with bedding, food pellets and a partition for 60 min and sleep deprived by cage tapping ('Sleep deprivation' session). (2) C57BL/6j mice were placed in a cage with a partition previously used by a CD-1 mouse for more than 5 days ('No resident' session). (3) The intruder mouse was placed in the resident cage while the

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intruder and resident mice were separated by the partition for 60 min ('No contact' session). Sleep deprivation by cage tapping was not conducted during the 'No resident' and 'No contact' sessions.

Vigilance state assessment based on EEG/EMG polygraphic recordings

One week after surgery, the mice were individually housed in experimental cages in an insulated soundproof recording chamber and connected to the EEG-EMG recording cables. Mice were habituated for 2 to 3 days before starting the polygraphic recordings. Before the SoD session, some mice were subjected to a 'Sleep deprivation' or 'No resident' session, followed by a 'No contact' session. Sessions were run every 3 or 4 days. Baseline was recorded on the day prior to each session (**Figure 1A**). The EEG/EMG signals were amplified, filtered (EEG 0.5-30 Hz; EMG 20-200 Hz), digitized at a sampling rate of 128 Hz together with locomotion signals detected by infrared sensors, and recorded using the data acquisition software SleepSign® (Kissei Comtec, Matsumoto, Japan). The vigilance states were classified offline in 10-s epochs of three stages, i.e., wakefulness, REMS and SWS by SleepSign® (ver 3.4). After the pre-assignment of stages by the software, the signal wave forms and EEG power spectrum of each epoch were manually examined and corrected when necessary. SWS was assigned to epochs with high amplitude of SWA (0.5-

4 Hz) of EEG and low EMG. REMS was characterized by theta (6-10 Hz)-dominant EEG

and silent EMG. Wakefulness was assigned according to EMG activity, locomotion and

low amplitude of EEG. Spectral analysis of EEG by fast Fourier transform was performed,

and the EEG power densities were calculated in the range of 0.5–25 Hz in 0.5-Hz bins.

SWA during SWS was calculated based on EEG power in the range of 0.5–4 Hz. In a 3-

hourly plot of SWA, the data were presented as percentages of the mean of the 12-h

baseline SWS during the dark period.

Behavioral analysis

Animal behaviors during 'No resident' and 'No contact' sessions were analyzed

using video recordings. Vigilance states and behaviors during wakefulness were scored

in 4-s epochs. Behaviors were scored as grooming, exploration (including ambulation,

rearing, digging, and sniffing), consumption (eating and drinking) or quiet waking, when

the behavior accounted for more than 50% of the epoch.

Blood sampling and corticosterone measurement

Blood sampling was performed by cardiac puncture. Mice were deeply

anesthetized with isoflurane and blood was collected with an EDTA (pH 8, adjusted with

NaOH) -coated 1 ml syringe with a 25 gauge needle. Blood sampling were conducted

immediately after the SoD sessions with 2, 4 or 8 defeats. Blood samples were obtained

within 2 min, which is rapid enough to ensure that the stress imposed by the blood-

sampling procedure did not affect the corticosterone levels in the plasma (Riley, 1960).

For the control group, blood was collected immediately after the 'No resident' session.

For basal corticosterone levels, blood was collected from undisturbed mice in their home

cages at zeitgeber time 12. The procedure of cardiac puncture was conducted by Mahesh

K. Kaushik in Yanagisawa/Funato laboratory due to a technical reason, and the applicant

served as an assistance. All the mice were used only once and not subjected to any

behavioral tests. Blood samples were immediately centrifuged at 10,000 rpm for 15 min

at 4°C. Plasma samples were collected and stored at -80°C until the assay was performed.

Plasma corticosterone was measured in duplicate wells using the DetectX® ELISA kit

(Arbor Assays, Ann Arbor, MI, USA). The mean of intra-assay coefficients of variations

(n=31) calculated from duplicate wells was 2.82%.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software (GraphPad

Software, La Jolla, CA, USA). All results are presented as mean ± standard error of the

mean (SEM). A paired two-tailed Student's *t*-test was used for statistical comparisons between paired groups (Figure 3, Figure 4A, Figure 6A, Figures 7A,B,C, Figure 8B, Table 2 and Table 4). Two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni's *post hoc* comparisons was used to analyze the sleep/wake profile (Figure 2) and EEG power (Figures 8B,C). One-way ANOVA followed by Bonferroni's (Figures 4B,C and Figure 5) or Dunnetts (Figure 6B and Figure 8B) *post hoc* comparisons were performed to compare groups of three or more. The Kruskal-Wallis test was used to analyze the data with significant variance heterogeneity assessed by Levene's test (Figure 4B). In all cases, $P < 0.05$ was considered significant.

Results

SoD stress strongly increases sleep

I established a mouse model of sleep alterations after acute SoD stress based on a resident-intruder paradigm, whereby a C57BL/6j male mouse was introduced as an intruder to a resident CD-1 male mouse selected for its high level of aggression (**Figure 1**). To prevent injury to the intruder mouse, the intruder and resident mice were separated by a partition. During a 1-h SoD session, the partition was removed multiple times and reinserted every time when the intruder mouse was attacked and showed submissive behavior. After the SoD session, the intruder mouse was returned to the home cage and EEG and EMG of the intruder mice were recorded.

First, I investigated the extent to which sleep was affected by different numbers of defeats during a 1-h session. Intruder mice were exposed to two, four or eight successive defeats. In each successive defeat, mice were in contact with the resident mice for 15.5 ± 1.5 s. I analyzed EEG and EMG recordings made after the SoD session or on the previous day (baseline). SWS strongly increased from the first 3 h after the sessions with two, four and eight defeats. The strong increase in SWS lasted until 6 h or 9 h after the sessions with two or four defeats, respectively. Accordingly, wakefulness was

decreased during the corresponding periods. A REMS increase was observed only between 6 and 9 h after the SoD session with four successive defeats (**Figure 2**, **Table 1**). When the first 3 h following the SoD sessions were analyzed in more detail, an increase in wakefulness only occurred during the first 10 min after the SoD sessions and SWS started to increase afterwards (**Figure 3**). Because most of the effects on sleep stages were observed within 9 h after the sessions, I compared the total amounts of 9 h following the SoD sessions (**Figure 4A**, **Table 2**). The total baseline amounts of the vigilance states during the same time period were not significantly different among groups (SWS: $P = 0.4914$, $F(2, 20) = 0.7364$, REMS: $P = 0.0761$, $F(2, 20) = 2.938$, Wake: $P = 0.3578$, $F(2, 20) = 1.083$). SWS significantly increased after SoD sessions, whereas wakefulness decreased accordingly. By contrast, REMS was only increased significantly after four defeats. Moreover, the calculated changes in the total amounts of SWS, REMS and wakefulness between baseline and the post-SoD 9-h periods revealed a significant difference in REMS only between four and eight times (**Figure 4B**, **Table 1**). Because variance in SWS or wakefulness was highly significantly different among groups, I performed Bonferroni's *post-hoc* comparisons on the absolute deviations from the mean (**Figure 4C**). The deviations were significantly lower only in the intruder group with four defeats. Finally, I studied corticosterone responses in the intruder mice after SoD as an

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indicator of stress due to activation of the HPA axis, the central stress response system (Mormède et al., 2007). Blood samples for measuring corticosterone levels were collected from undisturbed mice (to establish the baseline) and intruder mice immediately after the SoD sessions. A control session in a cage previously used by the resident mouse ('No resident') was also conducted. SoD sessions significantly increased corticosterone levels and it was significantly stronger compared with 'No resident' mice ($P < 0.0001$, $F(4, 26) = 24.5$, one-way ANOVA followed by Bonferroni's *post hoc* comparisons, **Figure 5**). These results may indicate that SoD causes stress in mice. However, there was no significant difference between SoD sessions with different numbers of successive defeats. These results suggested that the SWS increase observed after SoD stress is mostly independent of the numbers of defeats.

Specific versus non-specific effects of the SoD

To differentiate between the specific effects of SoD and non-specific effects of the SoD procedure (e.g., novelty or sleep deprivation), the sleep/wake behavior of mice after SoD stress was compared with that following other conditions, including sleep deprivation in a clean unused cage ('Sleep deprivation'), a cage previously used by the resident mouse ('No resident'), or the presence of the resident mouse separated by a

partition ('No contact'). Data of the control conditions were compared with those of four successive defeats, because SWS consolidation occurred with significantly less variation after this SoD procedure than the ones with two or eight defeats.

During the 'No resident' or 'No contact' session, the animals were spontaneously active and awake during most of the 1-h session (**Table 3**). 'Sleep deprivation' and 'No contact' sessions significantly increased SWS and decreased wakefulness during a 9-h period after the sessions (**Figure 6A**, **Table 4**); however, the SWS increase was smaller than that after SoD stress (**Figure 6B**). SWS was not changed after the 'No resident' session (**Figure 6A**). REMS also increased after the SoD and control procedures; however, there was only a significant difference between the baseline and experimental periods after SoD stress (**Figure 6A**). These results suggest that a significant part of the sleep changes after SoD can be attributed to non-specific sleep deprivation during the SoD session, i.e., about half of the increase of SWS and all of the increase of REMS may be a homeostatic sleep response to non-specific sleep deprivation.

SoD stress increases the number of SWS episodes

The sleep architecture and SWA were then analyzed after SoD session with four successive defeats. SoD stress significantly affected the episode number of SWS and

wakefulness for 9 h after the SoD session (SWS: $P = 0.0079$, Wake: $P = 0.0077$, paired

Student's t-test, **Figure 7A**). The mean duration of wake episodes decreased by 48.4% ±

6.9% ($P = 0.0035$, paired Student's t-test) compared with the baseline, whereas the

duration of the SWS or REMS episodes was not significantly different or slightly

increased, respectively (**Figure 7B**). The number of stage transitions from SWS to wake

and wake to SWS was significantly increased (SWS to Wake: $P = 0.0131$, Wake to SWS:

$P = 0.0076$, paired Student's t-test), whereas other stage transitions were not affected by

SoD stress (**Figure 7C**).

SoD stress increases EEG slow wave activity during SWS

To assess whether EEG activity was altered by SoD stress, I compared the

normalized EEG power spectrum of SWS for 30 min following the SoD session with

baseline SWS. EEG activity was significantly increased in the frequency range of 1–4 Hz

and decreased in the frequency range of 4.5–10 Hz during SWS (interaction $P < 0.0001$,

$F(49, 350) = 25.47$, two-way repeated-measures ANOVA followed by Bonferroni's *post*

hoc comparisons, **Figure 8A**). SWA in the frequency range of 0.5–4 Hz was then

calculated during the same period and compared with the 'Sleep deprivation', 'No

resident' and 'No contact' conditions. SWA was significantly increased in 'No resident',

'No contact' and SoD mice, although the increase of SWA in the SoD mice was significantly stronger than that in the 'No resident' mice, but not in the 'No contact' mice (P = 0.0176, $F(3, 20)$ = 4.258, one-way ANOVA followed by Dunnetts *post hoc* comparisons, **Figure 8B**). Finally, I calculated the 3-h SWAs in intruder mice for 12 h following SoD stress and found that the first 3-h SWA was significantly higher than the baseline SWA on the previous day (main effect P = 0.0005, $F(1, 28)$ = 15.66, interaction P = 0.0002, $F(3, 28)$ = 8.991, two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* comparisons, **Figure 8C**).

Discussion

The present study explored the effect of acute SoD stress on the sleep/wake cycle in mice. SWS was strongly promoted over 9 h after SoD stress. Corticosterone, as an indicator of stress, was significantly increased in the blood plasma by SoD, confirming that SoD induced stress in the mice. The increase in SWS was associated with more transitions from wakefulness to SWS without changing the mean episode duration of SWS.

The effect of acute SoD stress on subsequent sleep amount was reported in several studies. One study showed strong SWS increase in mice after acute SoD stress by interaction for 1 h with mice from the same strain (Meerlo and Turek, 2001). By contrast, another study using the same procedure showed little effect of SoD on the SWS amount (Vaanholt et al., 2003). Due to the lack of information about the number and duration of attacks of the resident mice against the intruder mice, it is hard to explain the difference between the studies. Moreover, it was also reported that wakefulness is increased in the first 3 hours after SoD followed by a sleep increase during later hours (Henderson et al., 2017). Highly aggressive male CD-1 mice were used in this study for the SoD of C57BL/6j mice during a 5-min interaction period followed by a 20-min period of

olfactory, visual, and auditory contact between the resident and intruder mice. The initial

increase of wakefulness in this study may be attributed to repeated painful attacks of the

CD-1 mice against smaller C57BL/6j mice. Alternatively, the duration of physical and

sensory stress (25 min) may be too short to induce sleep. Although variations in the

aggressive behavior of the resident mice may account to some extent for the differences

in the sleep/wake responses between the studies, sleep architecture, SWA and

corticosterone responses in socially defeated mice may also be influenced by other factors.

For example, laboratory environment and time-of-the-day when the conflict was

introduced may affect sleep and corticosterone parameters. Moreover, prior stress

experiences of the intruder mice such as early life stress during maternal care, fighting

between littermates or low social ranking in the litter can also be important factors for

outcomes after SoD stress (Wang et al., 2014; Peña et al., 2017). I designed a SoD stress

protocol to minimize behavioral variations during SoD. In this protocol, intruder mice

were separated from aggressive resident mice (CD-1 mice) when they exhibited

submissive behaviors rather than by setting a fixed duration of interaction with the

resident mice, preventing repeated and painful attacks against the submissive mouse.

Moreover, physical interaction was conducted two, four or eight times to maintain a high

level of SoD stress in the intruders during the session. I found that acute SoD stress,

introduced at the end of the light period, increases SWS shortly after the SoD session.

Moreover, the comparison of the specific effects of SoD and non-specific effects of the

SoD procedure showed that a large portion of the sleep changes after SoD stress can be

attributed to a homeostatic response to sleep deprivation. On the other hand, about half

of the SWS increase is likely a specific response to SoD.

I also observed a strong increase in SWA after the SoD session (**Figures 4E,F**),

consistent with previous reports in mice and rats (Meerlo et al., 1997; Meerlo and Turek,

2001; Kamphuis et al., 2015; Henderson et al., 2017). An increase in SWA is considered

the hallmark of homeostatic sleep need in response to sleep deprivation (Suzuki et al.,

2013; Dispersyn et al., 2017; Wang et al., 2018). The SWA increase, however, was

stronger after the SoD condition than under sleep-disrupting control conditions without a

CD-1 mouse. Moreover, SWS was still enhanced long after the SWA returned to the

baseline level. These observations suggest that SWA is also affected by social stress and

at least part of the SWS in response to SoD is independent of SWA.

Limitation of the study

I observed large variations in the SWS increase after the SoD sessions, especially

the ones with two and eight successive defeats (**Figures 2D,E**), where about half of the

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animals did not show strong SWS increase. It is well known that group-housed male mice

establish a social hierarchy (Wang et al., 2014). As I did not control the social ranking of

the intruder mice before the SoD session, it is possible that the SWS response of the

intruder mice is affected by their SoD resilience. Nevertheless, the variation of the SWS

increase after four successive defeats was significantly smaller than those of two and eight.

For some animals, two defeats may not have been enough to maintain high level of SoD

stress during the entire 1h session although the corticosterone level as an indicator of

stress was not different between groups. On the other hand, eight defeats might be too

frequent to observe SWS increase. Although injury was prevented in this study, the pain

caused by attacks in each physical contact may have blunted the SWS increase. Another

possibility is the difference of dissipation speed of the stress reactions after stronger stress.

It is reported that social conflict temporarily increases the blood corticosterone as well as

heart rate, blood pressure and body temperature, to the similar extent in both losers and

winners in rats, but the dissipation of those responses was slower in the losers (Koolhaas

et al., 2011, 2017). Thus, the difference of the stress intensity among different numbers

of physical contacts may be expressed in the dissipation speed of the stress reactions. In

some animals that experienced eight defeats, the prolonged stress reaction may have

promoted arousal directly or indirectly through the action of glucocorticoids (Hirotsu et

al., 2015). Thus, although corticosterone was measured only immediately after the stress

sessions in the current study, time-course measurement of corticosterone after stress may

better assess the stress intensity. In addition to corticosterone, sympathetic activities

expressed in such as heart rate, body temperature or blood adrenaline may also be helpful

to evaluate stress because those indicators have different temporal dynamics from

corticosterone (Koolhaas et al., 1997, 2017).

Although current study elucidated the specific effect of SoD stress by comparing

with appropriate controls, it is important to note that control sessions may also cause some

stress, which may influence the following sleep. It is reported that placing a mouse to an

unused clean cage causes mild stress which is assessed as an increase in blood

corticosterone (Xu et al., 2014). In addition, the restriction of the cage space by partition

during 'Sleep deprivation' or 'No resident' session can be another source of stress. In fact,

'No resident' session also increased the corticosterone level compared to the baseline

(**Figure 5**).

Mechanism of sleep response to SoD stress

The neuronal mechanisms of the sleep response to SoD stress are unknown.

Acute stress response involves various neurotransmitters including noradrenaline,

dopamine, orexin and glutamate (Anstrom et al., 2009; Gold, 2015; Grafe and Bhatnagar,

2018). Immunohistological staining of c-fos, the neuronal activity marker, revealed that SoD stress in rats activates the majority of the brain areas (Lkhagvasuren et al., 2014). Thus, the accumulation of the homeostatic sleep need is considered to be driven by enhanced neuronal activity during stress, which was observed as enhancement of SWA in this study. However, the brain areas regulating homeostatic sleep need is still unclear. Because various brain areas are activated by the stress, it is conceivable that SWS promotion after stress involves distinct mechanisms from those of sleep deprivation. Adenosine accumulation has been considered to be important in the recovery sleep after sleep deprivation, and phosphorylation of a series of neuronal proteins in the brain is reported to regulate the homeostatic sleep need. However, the brain areas and temporal dynamics of those mechanism during stress relative to sleep deprivation remain to be investigated. For example, accumulation of adenosine during wakefulness has been reported in cholinergic basal forebrain, hippocampus and cortex, where adenosine A_1 receptors are expressed, and A_1 receptors has been shown to be important in the homeostatic sleep response (Halassa et al., 2009; Bjorness et al., 2009, 2016). On the other hand, a subset of neurons in nucleus accumbens in the basal ganglia expresses adenosine A_{2A} receptors. A_{2A} receptor agonists administered to the brain induce a dramatic increase in sleep (Satoh et al., 1996, 1998, 1999), and arousal effect of caffeine,

a non-selective adenosine antagonist, is dependent on the A_{2A} receptors in the nucleus accumbens (Huang et al., 2005; Lazarus et al., 2011). Importantly, adenosine signaling on A_{2A} receptor has been associated with depressive symptoms induced by stress (Yamada et al., 2014; Domenici et al., 2019; van Calker et al., 2019). However, the role of endogenous adenosine produced during behavior in nucleus accumbens on sleep regulation is unknown.

The mesolimbic dopamine system plays an important role in the development of depression-related behaviors after repeated SoD stress (Russo and Nestler, 2013). The mesolimbic system comprises dopaminergic projections from the ventral tegmental area to the nucleus accumbens, an area critical for reward and motivation (Graybiel, 2008; Russo and Nestler, 2013), and is also connected to the medial prefrontal cortex, amygdala and hippocampus, allowing the system to integrate cognition, emotion and action (Floresco, 2015). Recently it was shown that the mesolimbic system plays an important role in the gating of sleep by motivated behavior (Eban-Rothschild et al., 2016; Oishi et al., 2017b, 2017c; Luo et al., 2018). Therefore, it is plausible that the mesolimbic system also mediates sleep responses to SoD stress.

Possible function of the sleep response after SoD stress

Acute stress responses induce behavioral, sympathetic and neuroendocrine changes in animals to facilitate a fight or flight response (Chrousos, 2009). Sympathetic and neuroendocrine responses, including the increase of body temperature and heart rate and secretion of noradrenaline, adrenaline and glucocorticoids, usually dissipate within hours after acute SoD in rodents (Koolhaas et al., 1997, 2017). Sleep is known to inhibit glucocorticoid secretion in healthy men (Weitzman et al., 1983), whereas REMS disinhibition and dysregulation of HPA axis are hallmarks of depression and chronic stress (Nollet et al., 2019; Steiger and Pawlowski, 2019). The increased SWS after SoD stress in this study may facilitate the termination of stress responses. Moreover, SWS induction may also be beneficial for restoration of brain homeostasis after the stress response (Tononi and Cirelli, 2003, 2006; Xie et al., 2013; Ding et al., 2016). By contrast, REMS is considered to have an important function in the processing of emotional memory of adverse experiences to promote emotional and mental recovery (Suchecki et al., 2012; Goldstein and Walker, 2014). Only a moderate REMS increase was observed during a period 6 to 9 h after SoD sessions with four defeats (**Figure 2**). Most, if not all, of the REMS increase, however, may be attributed to non-specific sleep deprivation during the SoD session and thus, a beneficial effect of REMS after SoD remains unclear.

Then, how SWS increase after the SoD stress can be adaptive in the natural

environment? Although not being defeated is more successful in gaining resources and

increasing reproduction opportunities, animals accept a lower social rank due to the

inability to dominate the opponent. This study investigated the sleep response on the

defeated animals. In wild situations, for the subordinate animals to be successful, they

presumably have to wait for a chance to dominate conspecifics by situational change or

have to find other possible resources through exploration. Thus, it seems especially

important for the subordinate animals to maintain health for future success and good

cognitive function to notice slight changes in the environment. The increase of SWS after

SoD may be beneficial to their health by normalizing their deviated homeostasis by the

stress. Moreover, because SoD stress increase the sleep need and sleep deprivation

impairs cognitive performance (Tononi and Cirelli, 2003, 2006; Alhola and Polo-Kantola,

2007), the SWS after SoD might also be important to restore cognitive performance such

as recognition, learning, memory retrieval, working memory or appropriate decision

making. In the future study, investigation of cognitive performance of defeated mice may

reveal roles of sleep after SoD stress (Dudchenko, 2004).

In the context of subordination, the reactive coping style seems to be more

successful because reactive animals are more flexible in their behavior and sensitive to

environmental changes. While it has been reported that reactive and proactive animals are

bimodally distributed in the feral population of house mice, laboratory strains of animals

do not show such distribution because the coping styles are considered genetically

decided through selection pressure (Koolhaas et al., 1999). It would be interesting to

investigate how sleep response to SoD is different between populations with distinct

coping styles (Van Oortmerssen et al., 1990; Benus et al., 1991).

Conclusion

This study revealed strong SWS responses to acute SoD stress in submissive male mice attempting to appease their aggressive counterpart, when painful attacks on the submissive mice were prevented. The SoD stress model will be useful for future study. One of the future interests will be the regulatory mechanism of sleep homeostasis during and after the stress. The role of sleep in the recovery of cognitive functions after acute stress is an important topic. Insomnia is often triggered by stress, and the disrupted sleep contributes to the exacerbation of psychiatric disorders. The investigation of the sleep homeostasis under stress may help understand the interrelation of sleep disruption and psychiatric disorders.

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Source

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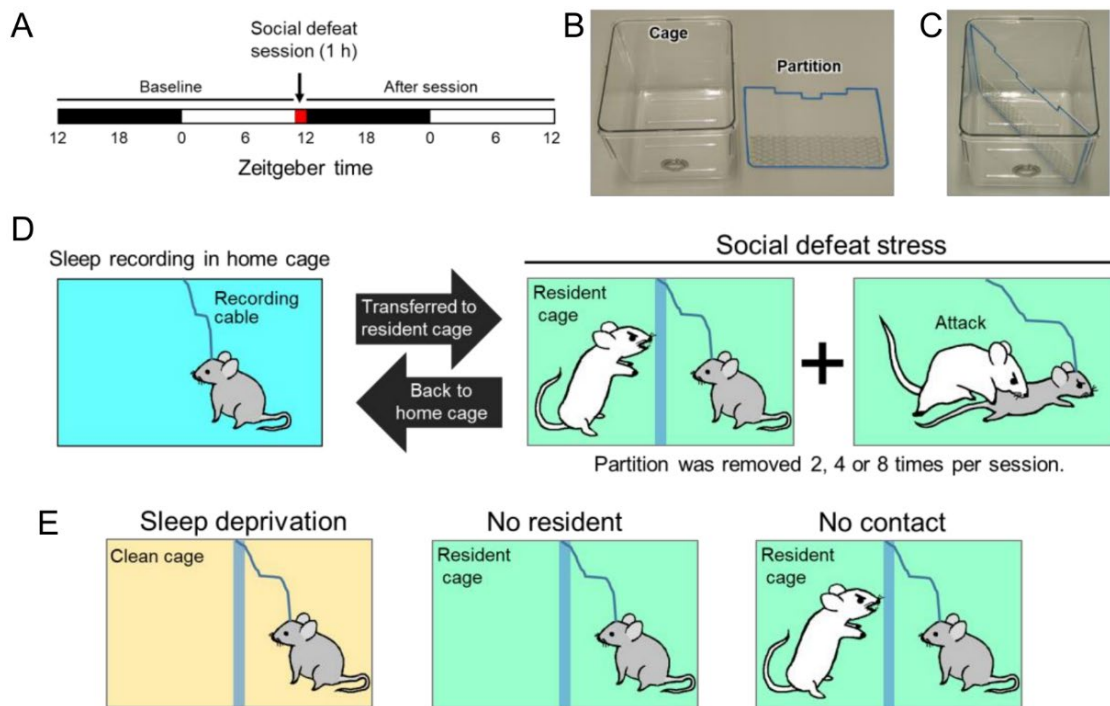
Figures and tables

Figure 1 Protocol for SoD stress based on a resident-intruder paradigm. (A) Schedule of sleep recording and SoD session. (B) Cage used for SoD or EEG/EMG recording and partition for diagonal separation of the cage. (C) Cage with inserted partition. (D) Schematic diagram of the SoD procedure. The intruder mouse was placed behind the partition of the resident cage. The partition was repeatedly removed for multiple defeat experiences of the intruder mouse by the resident mouse during a 1-h period. After the SoD session, the intruder mouse was returned to the home cage for sleep recording. (E) Control experiments to differentiate between the specific effects of SoD and non-specific effects of the SoD procedure. The mouse was placed in the cage for 1 h under each condition.
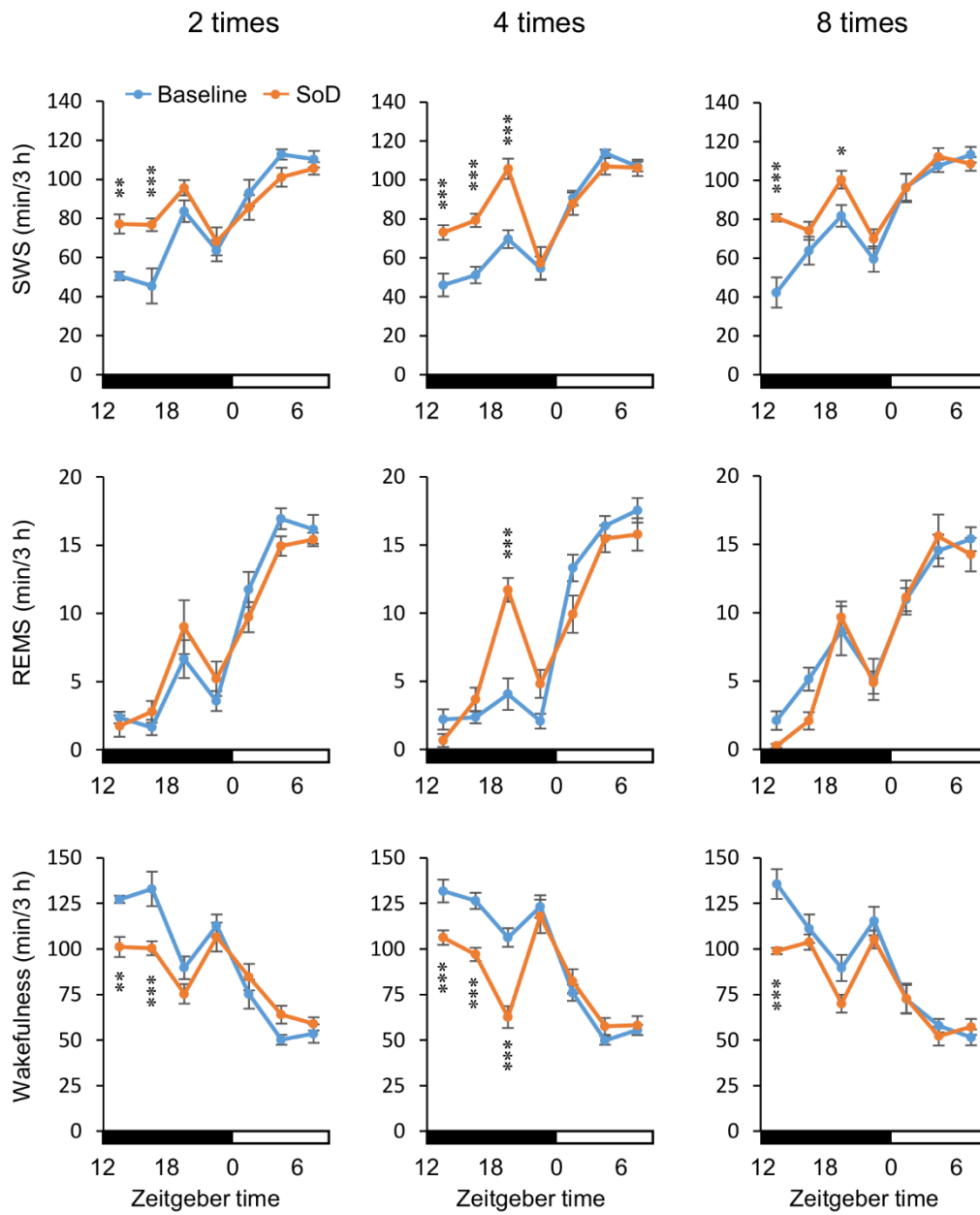
Figure 2 Time-course of SWS, REMS and wakefulness for 21 h after SoD sessions with two (n = 8), four (n = 8) or eight (n = 7) defeats. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with baseline, assessed by two-way repeated measures ANOVA followed by Bonferroni's *post hoc* comparisons.
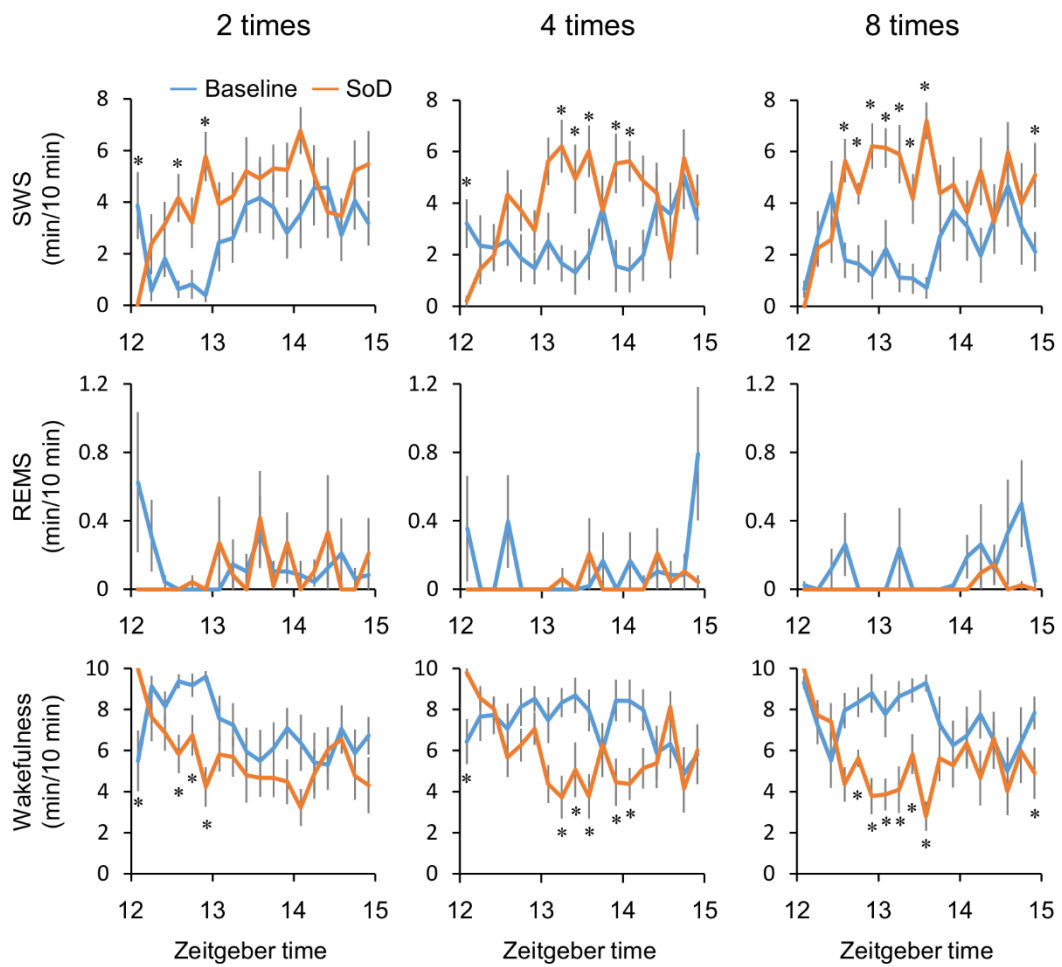
Figure 3 Time-course of SWS, REMS and wakefulness for first 3 h after SoD sessions.

*$P < 0.05$ compared with baseline, assessed by paired two-tailed Student's t-test.
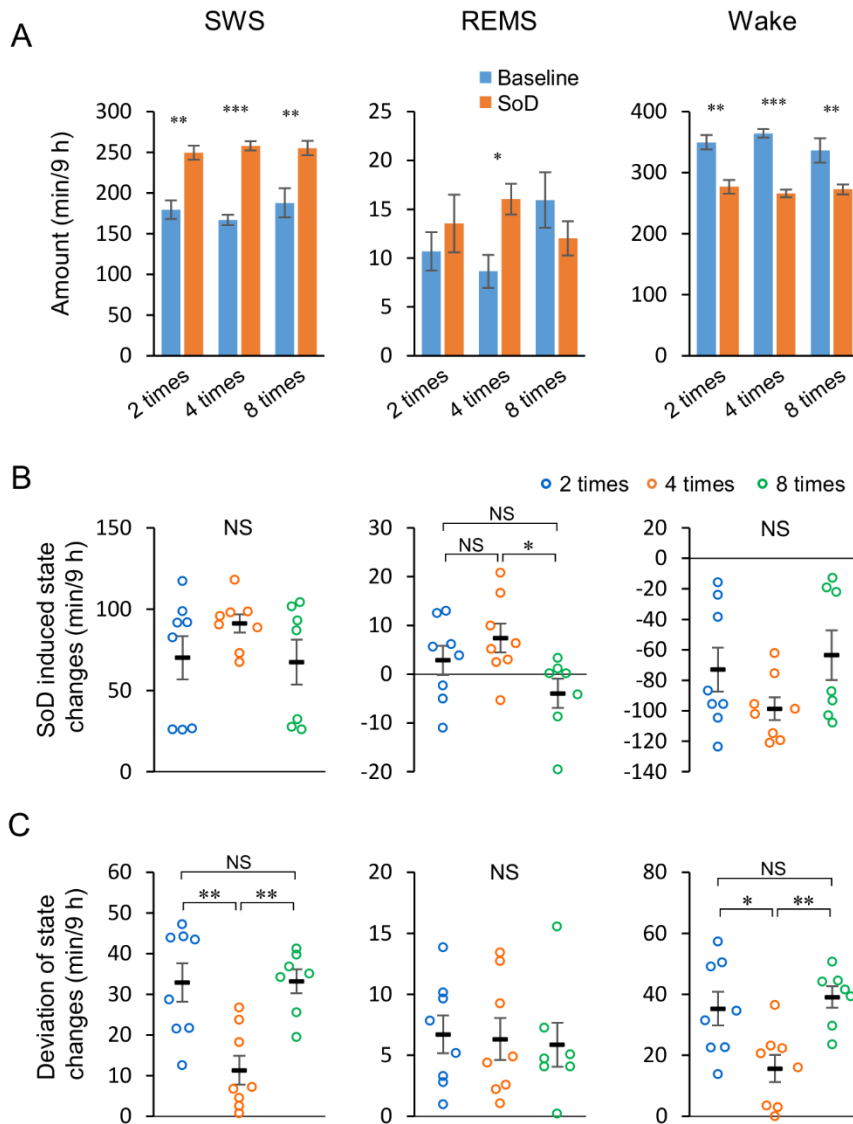
Figure 4 Effect of SoD on vigilance stages in total amounts (A) Total amounts of SWS, REMS and wakefulness for 9 h after SoD sessions. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with baseline, assessed by paired two-tailed Student's *t*-test. (B) Changes in total amounts of SWS, REMS and wakefulness for 9 h between baseline and after SoD sessions. *$P < 0.05$, compared between groups, assessed by the Kruskal–Wallis test (SWS and wakefulness) or one-way ANOVA (REMS) followed by Bonferroni's *post hoc*

comparisons. (C) Deviation of changes in total amounts of SWS, REMS and wakefulness.

The deviation was calculated by subtracting the mean from the experimental values of

each animal for changes in the total amounts of SWS and wakefulness for 9 h in each

condition. $*P < 0.05$ and $**P < 0.01$, compared between groups, assessed by one-way

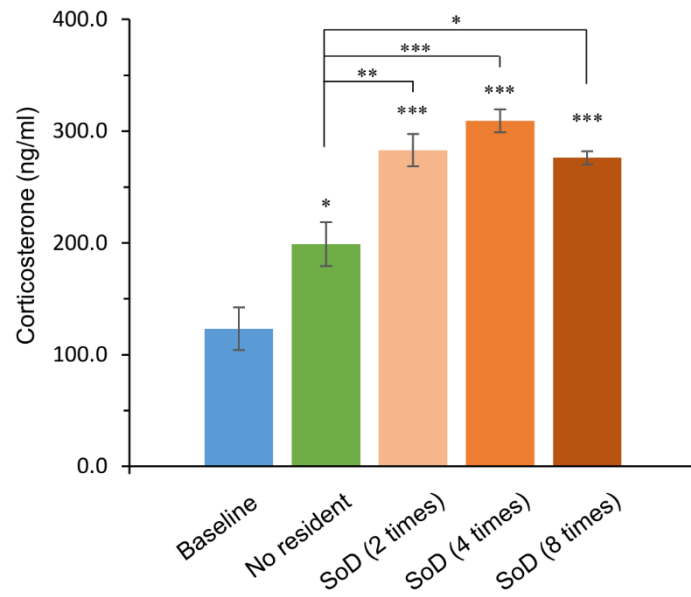ANOVA followed by Bonferroni's *post hoc* comparisons. NS, not significant

Figure 5 Blood plasma corticosterone levels from undisturbed mice (n = 6) at zeitgeber

time 12 of the baseline day or mice after a 'No resident' (n = 7) or SoD session (n = 6).

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared between groups, assessed by one-way

ANOVA followed by Bonferroni's *post hoc* comparisons.

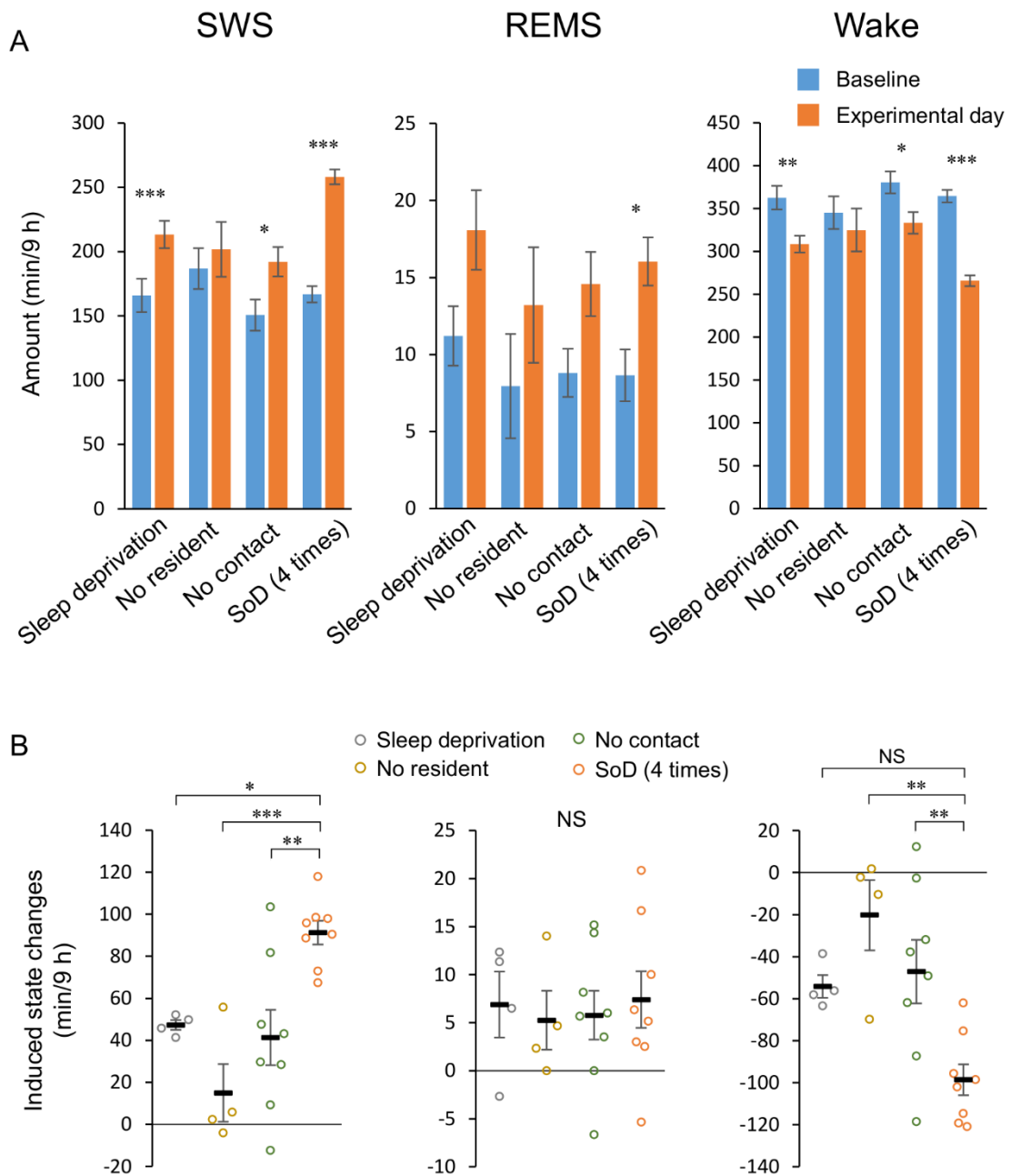**Figure 6** SoD is essential for a strong SWS increase. (A) Total amounts of SWS, REMS and wakefulness for 9 h after a 'Sleep deprivation' (n = 4), 'No resident' (n = 4), 'No contact' (n = 8) or SoD (n = 8) session with four defeats. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with baseline, assessed by paired two-tailed Student's t-test. (B) Changes in the total amounts of SWS, REMS and wakefulness for 9 h between baseline

and experimental days. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with SoD,

assessed by one-way ANOVA followed by Dunnett's *post hoc* comparisons. Data are
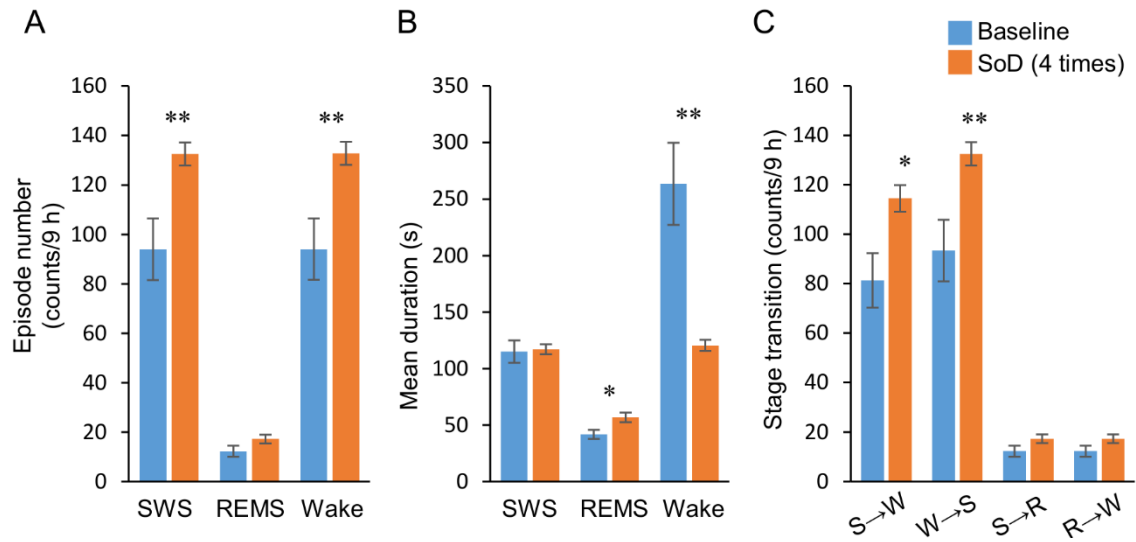
presented as means. NS, not significant

Figure 7 Sleep architecture after SoD stress. Episode numbers (A), mean episode durations (B) and numbers of stage transitions (C) for 9 h after SoD session with four defeats. *$P < 0.05$ and **$P < 0.01$ compared with baseline, assessed by paired two-tailed Student's *t*-test.
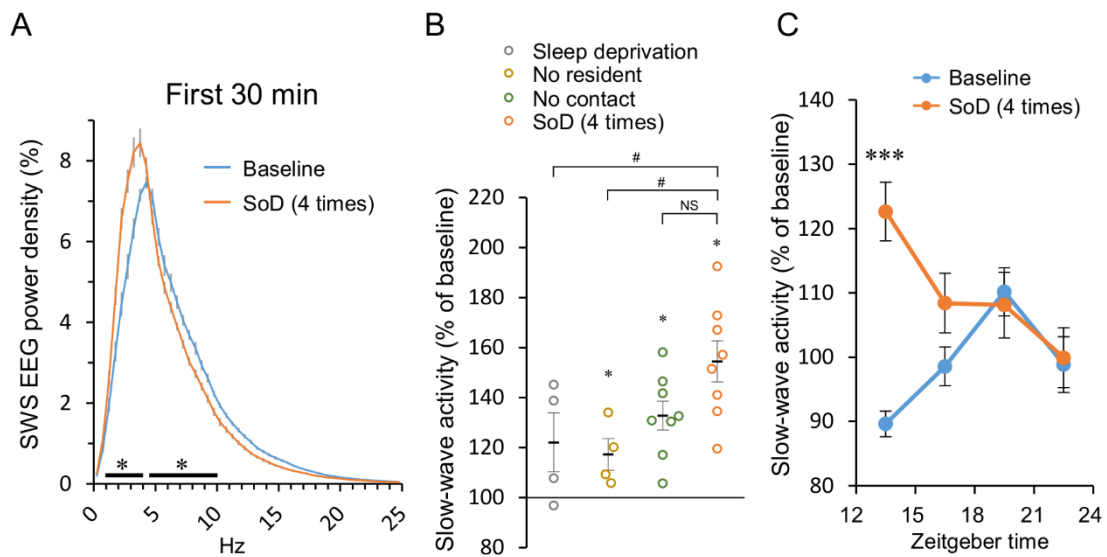
Figure 8 EEG power during SWS after SoD stress. (A) Power density of EEG during SWS for 30 min after SoD session with four defeats or onset of the dark phase on the baseline day. *$P < 0.05$ compared with baseline, assessed by two-way repeated measures ANOVA followed by Bonferroni's *post hoc* comparisons. (B) Changes in the SWA (0.5–4 Hz) relative to the baseline for 30 min after a 'Sleep deprivation', 'No resident', 'No contact' or SoD session. *$P < 0.05$ compared with baseline, assessed by paired two-tailed Student's *t*-test, #$P < 0.05$ compared with SoD, assessed by one-way ANOVA followed by Dunnett's *post hoc* comparisons. (F) Time-course of SWA changes after SoD session. ***$P < 0.001$ compared with baseline, assessed by two-way repeated measures ANOVA followed by Bonferroni's *post hoc* comparisons. NS, not significant

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Table 1 Statistics of data shown in Figures 2A,B and 3B.

Two-way repeated measures ANOVA for Figure 2A			
		Main effect	Interaction
SoD (2 times)	SWS	$P = 0.0057$, $F(1, 49) = 8.359$	$P < 0.0001$, $F(6, 49) = 6.405$
n=8	REMS	$P = 0.9359$, $F(1, 49) = 0.006532$	$P = 0.1535$, $F(6, 49) = 1.65$
	Wakefulness	$P = 0.0124$, $F(1, 49) = 6.744$	$P < 0.0001$, $F(6, 49) = 5.89$
SoD (4 times)	SWS	$P < 0.0001$, $F(1, 49) = 33.97$	$P < 0.0001$, $F(6, 49) = 10.76$
n=8	REMS	$P = 0.2284$, $F(1, 49) = 1.488$	$P < 0.0001$, $F(6, 49) = 8.931$
	Wakefulness	$P < 0.0001$, $F(1, 49) = 29.37$	$P < 0.0001$, $F(6, 49) = 11.06$
SoD (8 times)	SWS	$P < 0.0001$, $F(1, 42) = 20.49$	$P = 0.0007$, $F(6, 42) = 4.931$
n=7	REMS	$P = 0.2984$, $F(1, 42) = 1.109$	$P = 0.3936$, $F(6, 42) = 1.074$
	Wakefulness	$P = 0.0005$, $F(1, 42) = 14.38$	$P = 0.0049$, $F(6, 42) = 3.698$
One-way ANOVA or Kruskal-Wallis test for Figure 2D and Figure 3B			
		One-way ANOVA or Kruskal-Wallis test	Levene's test
Fig. 2D	SWS	$P = 0.5716$, $H = 1.118$	$P = 0.0004$, $F(2,20) = 11.69$
	REMS	$P = 0.0481$, $F(2,20) = 3.546$	$P = 0.9844$, $F(2,20) = 0.01575$
	Wakefulness	$P = 0.2035$, $H = 3.184$	$P = 0.0025$, $F(2,20) = 8.239$
Fig. 3B	SWS	$P = 0.0006$, $F(3,20) = 8.986$	$P = 0.0838$, $F(3,20) = 2.56$
	REMS	$P = 0.9563$, $F(3,20) = 0.1049$	$P = 0.8879$, $F(3,20) = 0.2106$
	Wakefulness	$P = 0.0026$, $F(3,20) = 6.713$	$P = 0.1492$, $F(3,20) = 1.981$

Table 2 Total amounts (minutes, mean ± SEM) of SWS, REMS and wakefulness for 6, 9, 12 and 24 h after SoD sessions with two, four or eight defeats.

SWS (min)		SoD (2 times) n=8	SoD (4 times) n=8	SoD (8 times) n=7
6h	Baseline	95.8 ± 8.9	97.2 ± 6.1	106.1 ± 14.5
	After session	153.9 ± 6.7***	152.4 ± 5.0***	154.9 ± 5.4**
9h	Baseline	179.5 ± 11.4	166.8 ± 6.3	187.8 ± 17.9
	After session	249.6 ± 8.7**	258.0 ± 5.8***	255.2 ± 8.9**
12h	Baseline	243.0 ± 12.6	221.5 ± 9.5	247.2 ± 18.3
	After session	317.8 ± 10.8**	315.3 ± 11.5***	325.1 ± 12.7***
24h	Baseline	655.0 ± 14.9	638.8 ± 12.3	663.6 ± 16.5
	After session	712.3 ± 13.0**	725.3 ± 16.7***	735.5 ± 14.3**

REMS (min)		SoD (2 times) n=8	SoD (4 times) n=8	SoD (8 times) n=7
6h	Baseline	4.0 ± 0.9	4.6 ± 1.0	7.3 ± 1.3
	After session	4.5 ± 1.3	4.3 ± 1.2	2.4 ± 0.8*
9h	Baseline	10.7 ± 2.0	8.6 ± 1.7	16.0 ± 2.8
	After session	13.5 ± 3.0	16.0 ± 1.6*	12.0 ± 1.8
12h	Baseline	14.3 ± 2.5	10.7 ± 1.8	21.1 ± 2.5
	After session	18.8 ± 3.6	20.9 ± 1.5**	16.9 ± 2.0
24h	Baseline	70.9 ± 1.6	72.3 ± 3.8	77.3 ± 2.6
	After session	73.6 ± 3.9	79.9 ± 2.6	69.8 ± 3.7

Wakefulness (min)		SoD (2 times) n=8	SoD (4 times) n=8	SoD (8 times) n=7
6h	Baseline	260.1 ± 9.3	258.3 ± 6.8	246.7 ± 15.4
	After session	201.5 ± 7.8***	203.3 ± 5.4***	202.7 ± 4.8*
9h	Baseline	349.9 ± 12.0	364.6 ± 7.2	336.3 ± 20.0
	After session	276.9 ± 11.4**	265.9 ± 6.4***	272.7 ± 8.2**
12h	Baseline	462.7 ± 14.0	487.8 ± 10.6	451.7 ± 20.1
	After session	383.4 ± 13.7**	383.9 ± 12.0***	378.0 ± 11.7**
24h	Baseline	714.1 ± 14.9	728.9 ± 13.7	699.0 ± 17.2
	After session	654.1 ± 16.1**	634.9 ± 18.0**	634.7 ± 11.3**

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with baseline, assessed by paired two-tailed Student's t-test.

Table 3 Percentage of animal behaviors (during wakefulness), SWS and REMS during

the 'No resident' and 'No contact' sessions.

Behavior (%)	No resident n=4	No contact n=8
Quiet waking	2.0 ± 0.8	3.9 ± 2.8
Grooming	14.5 ± 2.4	7.0 ± 1.5
Exploration	70.4 ± 6.9	79.2 ± 6.6
Consumption	3.1 ± 1.0	5.0 ± 0.8
SWS	9.6 ± 6.8	4.9 ± 4.5
REMS	0.3 ± 0.3	0.0 ± 0.0

Table 4 Total amounts (minutes, mean ± SEM) of SWS, REMS and wakefulness for 6, 9,

12 and 24 h after 'Sleep deprivation', 'No resident' and 'No contact' sessions.

SWS (min)		Sleep deprivation n=4	No resident n=4	No contact n=8
6h	Baseline	94.4 ± 10.9	103.1 ± 9.9	93.7 ± 8.7
	After session	132.0 ± 12.8**	118.7 ± 18.5	119.9 ± 7.0**
9h	Baseline	166.0 ± 12.9	186.7 ± 15.9	150.6 ± 12.2
	After session	213.3 ± 10.7***	201.7 ± 21.2	192.0 ± 11.5*
12h	Baseline	228.3 ± 19.9	229.3 ± 18.0	214.4 ± 12.6
	After session	263.8 ± 23.8**	239.9 ± 17.3	242.8 ± 10.6
24h	Baseline	619.6 ± 25.0	616.7 ± 8.4	623.1 ± 10.7
	After session	666.8 ± 37.3*	648.1 ± 13.7	653.9 ± 15.3
REMS (min)		Sleep deprivation n=4	No resident n=4	No contact n=8
6h	Baseline	3.8 ± 1.3	3.7 ± 1.2	4.8 ± 1.0
	After session	8.6 ± 0.6	6.4 ± 1.9	7.0 ± 1.2
9h	Baseline	11.2 ± 1.9	8.0 ± 3.4	8.8 ± 1.6
	After session	18.1 ± 2.6	13.2 ± 3.7	14.6 ± 2.1
12h	Baseline	15.6 ± 2.1	9.4 ± 3.7	12.7 ± 1.9
	After session	21.6 ± 2.4	16.3 ± 3.3	17.8 ± 2.0
24h	Baseline	77.2 ± 3.0	61.2 ± 2.2	70.6 ± 2.8
	After session	83.0 ± 3.3	74.7 ± 4.4	79.4 ± 3.9**
Wakefulness (min)		Sleep deprivation n=4	No resident n=4	No contact n=8
6h	Baseline	261.8 ± 11.5	253.2 ± 11.0	261.6 ± 9.0
	After session	219.4 ± 12.4***	235.0 ± 20.3	233.1 ± 7.7**
9h	Baseline	362.8 ± 13.7	345.3 ± 18.9	380.5 ± 12.8
	After session	308.7 ± 9.8**	325.1 ± 24.9	333.4 ± 12.7*
12h	Baseline	476.1 ± 20.2	481.3 ± 21.4	492.9 ± 13.3
	After session	434.6 ± 22.7**	463.8 ± 20.1	459.4 ± 11.6
24h	Baseline	743.2 ± 23.1	762.1 ± 6.3	746.3 ± 9.8
	After session	690.2 ± 39.8	717.2 ± 16.6	706.7 ± 15.7*

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with baseline, assessed by paired two-

tailed Student's t-test.