voxels, see Results) that exhibited a significant difference in fear vs. neutral contrast between the SD and SC sessions was used as the seed region of the connectivity analysis. Based on a previous study hypothesizing that sleep debt weakens the functional connectivity between the amygdala and the mPFC adjacent to the ACC [14], we placed the ROI in the mPFC region for the functional connectivity analysis and searched for the area that exhibited greater connectivity with the seed region in the SC session than that in the SD session. Using the WFU PickAtlas software in the SPM Toolbox, a mask for the ACC/mPFC region was generated based on AAL (by combining 'anterior cingulate' and 'medial frontal gyrus'), and each voxel in the mask was analyzed. The functional connectivity between the amygdala and the ACC/mPFC was analyzed using the CONN tool (Alfonso Nieto-Castanon, http://www.alfnie.com/software/conn) of SPM8.

Using GLM, voxels that were activated in relation to the BOLD signals in the seed region were extracted. Head motions and the hypothetical hemodynamic response to the main event (confounding effects of stimulus-locked transients [42]) and to the target were used as regressors, and the range of the bandpass-filter was set at 0.008–0.09 Hz. Connectivity contrasts thus created were used in first-level and second-level analyses, as in the analysis described earlier in the 'fMRI data analysis' section. Data were considered significant if p was less than 0.001 and the number of continuous voxels forming a cluster was greater than 5. Because of the larger size of the mask in the MPFC/ACC region, the small volume correction was not performed in functional connectivity analysis.

Correlation analysis between mood/sleep changes and fMRI data

Differences in the values between sessions were calculated for subjective mood (scores from the STAI-S and POMS questionnaires), $SWS_{2\ h}$, and $\delta_{2\ h}$. The contrast values between the sessions were also calculated for 1) amygdala activation and 2) the intensity of functional connectivity between the left amygdala and the ventral anterior cingulate cortex ($FC_{amg\text{-}vACC}$) in the fear vs. neutral condition under conscious presentation. Correlations between these psychometric and imaging contrast values were analyzed.

Clusters used in the analysis were the left amygdala, which showed differential activation between the SD and SC sessions, and the vACC, which showed different degrees of functional connectivity with the amygdala, as the seed region, between the sessions. Marsbar software (Matthew Brett, http://marsbar.sourceforge.net/marsbar.pdf) was used to calculate the mean contrast values in a cluster.

Statistics

The SPSS PASW Statistics 18 software package was used in statistical analysis. Differences in questionnaire scores, PSG data, and values of δ power between the SD and SC sessions were analyzed using the two-tailed t-test. Results are expressed as mean ± SD. Between-subjects test was performed by calculating Pearson's product moment correlation coefficient. Except for the analysis of functional brain activity, data were considered significant at $p<0.05$.

Results

Sleep time regulation

From the actigraph data, mean sleep time over the entire 5-day period in the SC and SD sessions was 8.09±0.35 h (8 h 5 min±21 min) and 4.60±0.54 h (4 h 36 min±32 min), with significantly fewer sleep hours (3.48±0.54 h, or 3 h 29 min±32 min) in the SD session [$t(13)=24.17$, $p<0.001$].

Sleepiness and mood states

Table 1 shows sleepiness and mood states associated with the SC and SD sessions. SSS and STAI-S scores for the SD session were significantly higher than those for the SC session, whereas no significant session-related differences were seen in POMS subscale scores.

PSG/delta wave power data

Sleep parameters and the analysis results are shown in Table 2. Compared with the SC session, the duration of TST, Stage 1, Stage 2, and REM were significantly shorter in the SD session; however, no differences were observed with Stage 3+4 or $SWS_{2\ h}$. As a result, the SD session had significantly higher %SWS and SE and significantly shorter SL. In addition, $\delta_{2\ h}$ for the SD session was significantly higher than that for the SC session.

Button Response

No significant session-related differences were seen in either the number or the mean time of responses (SC = 11.63±0.6, SD = 11.38±1.16, SC = 596.98±0.153.43, SD = 608.28±115.34, respectively).

fMRI activation

Comparison of fear vs. neutral contrasts for the conscious condition revealed significantly greater activation of the left amygdala in the SD session than in the SC session [peak MNI coordinates x = −14, y = 4, z = −18, $t(13)=-5.60$, FWE $p<0.05$ small volume correction] (Fig. 3; Table 3). Even though the activation of the right amygdala in the SD session was also higher than that in the SC session, it did not reach the significance level set for the analysis[peak MNI coordinates x = 18, y = 2, z = −18, $t(13)=-3.41$, $p=0.002$]. With regard to happy vs. neutral contrasts, the amygdala showed no session-related differences in activation [left amygdala, x = −14, y = 4, z = −14, $t(13)=-3.04$; right amygdala, x = 16, y = 2, z = −16, $t(13)=-1.90$].

Under the non-conscious condition, no significant differences in amygdala activation between the sessions was observed with fear vs. neutral contrasts [left amygdala; x = −14, y = −8, z = −16,

Table 1. Subjective sleepiness and mood state scores for the sleep control (SC) and sleep debt (SD) sessions; t and p-values for SC vs. SD with the paired t-test.

	SC session	SD session	t	p
SSS	2.14 (0.66)	3.21 (1.05)	−3.51	<0.01
STAI-state	35.64 (6.21)	39.43 (4.86)	−2.74	<0.05
POMS Vigor	53.54 (10.02)	53.54 (11.57)	0.00	N.S.
POMS Depression	47.76 (9.00)	48.13 (8.71)	−0.40	N.S.
POMS Anger-Hostility	42.84 (4.52)	42.24 (6.18)	0.60	N.S.
POMS Fatigue	45.94 (6.61)	48.13 (7.48)	−1.30	N.S.
POMS Tension-Anxiety	46.37 (10.57)	47.05 (9.92)	−0.46	N.S.
POMS Confusion	48.12 (8.07)	49.94 (9.32)	−0.99	N.S.

Data are expressed as mean (standard deviation) values; SSS, Stanford Sleepiness Scale; STAI, State-Trait Anxiety Inventory.
POMS, Profile of Mood States.
Degrees of freedom (df) = 13.
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Table 2. Values of spectral analysis and sleep variables for the sleep control (SC) and sleep debt (SD) sessions; *t* and *p*-values for SC vs. SD with the paired *t*-test.

	SC session	SD session	*t*	*p*
TST (min)	446.7 (22.5)	233.5 (7.5)	38.71	<0.001
Stage1 (min)	35.8 (20.0)	10.8 (8.2)	5.31	<0.001
Stage2 (min)	236.9 (35.3)	107.3 (28.9)	15.25	<0.001
Stage3 (min)	40.0 (11.3)	39.6 (15.4)	0.13	N.S.
Stage4 (min)	20.9 (25.8)	24.1 (25.8)	−1.52	N.S.
SWS (min)	60.8 (28.7)	63.7 (26.7)	−0.70	N.S.
REM (min)	113.1 (21.2)	51.7 (17.1)	11.09	<0.001
$SWS_{2\ h}$ (min)	37.5 (18.6)	41.4 (18.4)	−1.27	N.S.
Sleep latency (min)	19.3 (23.5)	3.3 (4.9)	2.90	<0.05
%SWS (%)	13.7 (6.7)	27.3 (11.6)	−7.21	<0.001
%REM (%)	25.4 (4.7)	22.2 (7.2)	1.80	N.S.
Sleep efficiency (%)	93.0 (4.7)	97.1 (3.1)	−3.46	<0.01
$Delta_{2\ h}$ (uV^2/min)	1340 (275)	1495 (312)	−3.108	<0.01

Data are expressed as mean (standard deviation) values; SC, sleep control; SD, sleep debt; SWS, slow wave sleep.
$SWS_{2\ h}$, slow wave sleep of first 2 h from sleep onset; $Delta_{2\ h}$, Delta wave power (0.5–4 Hz) of first 2 h from sleep onset.
Degrees of freedom (df) = 13.
doi:10.1371/journal.pone.0056578.t002

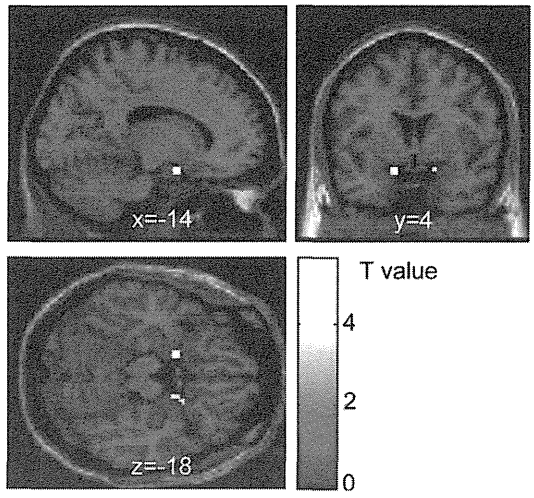

Figure 3. Difference in amygdala activation between the sleep control (SC) and sleep debt (SD) sessions. The map shows significantly greater activation in response to fearful face stimuli in the SD than SC session. Significant differences were seen in the left amygdala, peak MNI coordinate (*x*, *y*, *z*) = (−14, 4, −18) mm, $T(13) = 5.60$, $p = .0001$, $k = 8$ contiguous voxels. A similar trend was also observed in the right amygdala, (*x*, *y*, *z*) = (18, 2, −18) mm, $T(13) = 3.41$, $p = .0005$, $k = 7$. Significant clusters are rendered on a T1 anatomical referential image displayed in neurological convention, with the left side corresponding to the left hemisphere. The clusters shown are thresholded with a lenient alpha level ($p<0.01$, $k>5$) for visualization purposes. MNI, Montreal Neurological Institute template.
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$t(13) = -3.05$; right amygdala, x = 24, y = −4, z = −12, $t(13) = -3.08$] or happy vs. neutral contrasts [left amygdala; x = −22, y = −8, z = −14, $t(13) = -2.26$; right amygdala, x = 26, y = −6, z = −16, $t(13) = -2.60$].

We performed the analysis of functional connectivity using only the conscious and fear conditions that showed differential activation between the SD and SC sessions.

fMRI functional connectivity

Comparative analysis of fear vs. neutral contrasts for the conscious condition revealed that, compared with the SC session, $FC_{amg\text{-}vACC}$ was significantly diminished in the SD session [x = 14, y = 32, z = −4, $t(13) = 4.77$] (Fig. 4; Table 3).

Analysis of all of the task results from the SD and SC sessions showed a significantly negative correlation between the activation of left amygdala and $FC_{amg\text{-}vACC}$ [$r(13) = 0.63$, $p<0.001$] (Fig. 5).

Correlations between mood/sleep changes and fMRI data

Sleep debt-related cross-correlation between left amygdala activation, $FC_{amg\text{-}vACC}$, and changes in mood and sleep states are shown in Table 4. Changes of $FC_{amg\text{-}vACC}$ between sessions ($\Delta FC_{amg\text{-}vACC}$) were negatively correlated with the changes of degree of sleep debt ($\Delta SWS_{2\ h}$ and $\Delta\delta_{2\ h}$) as well as mood changes (ΔSTAI-S (Fig. 6), ΔPOMS Tension-Anxiety, and ΔPOMS Confusion). On the other hand, no significant correlations were observed between Δamygdala activation and any of the parameters of the changes in mood or sleep states.

Discussion

The results of this study revealed that sleep debt caused by having just a few hours of sleep for 5 days (3 h 29 min/day of sleep restriction compared with the SC session) increased the activity of the left amygdala in response to a fear facial image. In contrast, a happy facial image did not change the activity. Functional connectivity analysis demonstrated that the levels of $FC_{amg\text{-}vACC}$ (left amygdala-vACC functional connectivity) were lower in participants with higher degrees of sleep debt ($\Delta SWS_{2\ h}$ and $\Delta\delta_{2\ h}$). The most important and novel finding in this paper is that declines in $FC_{amg\text{-}vACC}$ were correlated with left amygdala activation and subjective mood deterioration (higher STAI-S and POMS scores). These findings strongly suggest that down-regulation of the amygdala by the vACC and subsequent activation of the amygdala in response to negative emotional stimuli are involved in intensified physiological and psychological responses [10,11,12] and mood deterioration [13,28] due to unpleasant emotional stimuli during sleep debt.

This interpretation is supported by a series of studies. The amygdala is thought to play an important role in the expression of negative emotions [43,44,45]. Facial expressions of fear were found to activate the amygdala even in healthy individuals with normal sleep [46,47], and such activation is reportedly more prominent in individuals with depression and anxiety disorders [48,49,50]. Moreover, the amygdala has a strong functional and anatomical connection with the mPFC region [51], and the strength of this functional connection is correlated with the degree of subjective emotional suppression and reappraisal of negative affect. [12,52,53].

According to previous studies [12,14], overnight total sleep deprivation diminishes the functional connectivity between the amygdala and the mPFC. In the present study, even a short-term, continuous and accumulating sleep debt that can occur in everyday life clearly resulted in reduced functional connectivity between the amygdala and the mPFC, and more specifically the vACC.

Table 3. Anatomical coordinates for regions of significant difference between the sleep control and sleep deprivation session compared with fear vs. neutral contrast.

Area		BA	MNI x	y	z	*t*	Z	*p*	Cluster k*
SD>SC Activity in amygdala mask									
Left	Amygdala	34	−14	4	−18	5.6	3.93	<0.001	9
SC>SD Functional connectivity with amygdala in ACC/MPFC mask									
Right	Anterior Cingulate	32	14	32	−4	4.77	3.56	<0.001	9

SC, sleep control session; SD, sleep debt session.
Cluster k* $p<0.001$ uncorrected threshold.
Degrees of freedom (df) = 13.
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More importantly, subjective mood changes (increased anxiety) following short sleep were correlated with diminished $FC_{amg\text{-}vACC}$, but not with the change in the activation in the amygdala (Δamygdala activation) itself. This may indicate that diminished synchronization between the amygdala and the vACC plays a more important role than the extent of the event-related local activation in the amygdala, for stabilizing increased anxiety evoked by an unpleasant emotional stimulus. Some models of amygdala functionality [53,54] suggest that the magnitude of the local activity in the amygdala does not play a direct role in modulating the mood states of individuals, but the functional connectivity between the amygdala and the ventral mPFC correlates with STAI state score during resting state fMRI [55]. In our functional connectivity analysis, the main event-related hemodynamic response covaries with the seed-related (amygdala) activity in the GLM model of functional connectivity analysis; namely, main event-related immediate reactivity was regressed in our functional connectivity results (see Friston et al., 1997 [42] for the detailed process). Therefore, $FC_{amg\text{-}vACC}$ in this study does not include simple event-related 'co-activation' between two regions, but finer event-unrelated synchronization between two regions beyond the local activities in two regions. This is why the local amygdala activity and the functional connectivity did not correlate with the psychological measurements in the same way; namely, the sustained anxiety (mood) state correlated with the $FC_{amg\text{-}vACC}$, but not with the event-related local reactivity of the amygdala. This interpretation is supported by the diminished functional connectivity between the amygdala and the vACC or ventral mPFC, regardless of the amygdala's activity, in individuals with social anxiety disorder, in those with the s allele of the serotonin transporter gene and who thus have a high risk of depression, and in those with schizophrenia [56,57,58].

Although it has been shown that positive emotional stimuli also activate the amygdala [54,55], happy facial expressions did not significantly alter amygdala activity during sleep debt in the present study. This suggests that functional changes in the amygdala and $FC_{amg\text{-}vACC}$ during sleep debt become more apparent when negative emotional stimuli are presented. Overnight total sleep deprivation reportedly induces activation of the

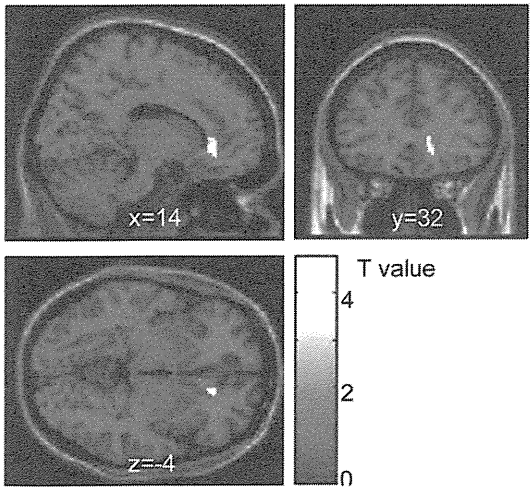

Figure 4. Difference in functional connectivity between sleep control (SC) and sleep debt (SD) sessions. The map shows greater functional connectivity between the left amygdala and other voxels in the brain in SC than SD session. Significant differences were found in the vACC, peak MNI coordinate (*x*, *y*, *z*) = (14, 32, −4) mm, $T(13) = 4.77$, $p = .0001$, $k = 9$ contiguous voxels. The significant cluster with a stronger connection with the left amygdala is rendered on a T1 anatomical referential image displayed in neurological convention, with the left side corresponding to the left hemisphere. The clusters shown are thresholded with a lenient alpha level ($p<0.01$, $k>5$) for visualization purposes. MNI, Montreal Neurological Institute template; vACC, ventral anterior cingulate cortex.
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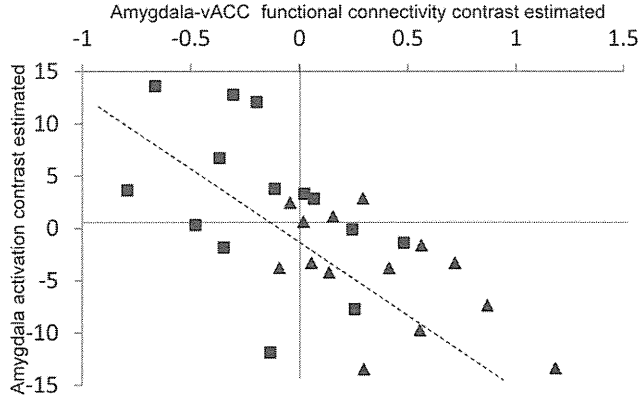

Figure 5. Correlation between amygdala activation and amygdala–vACC functional connectivity. Amygdala activation in response to fearful facial stimuli was negatively correlated with amygdala-vACC functional connectivity, $r(13) = .64$, $p = .0001$. The selected seed region within the amygdala was a cluster that showed greater functional connectivity with vACC in the SC than SD condition ($p<0.001$, uncorrected). Data from the SC and SD sessions were combined and plotted in one graph but differently colored; SD data in squares, SC in triangles. vACC, ventral anterior cingulate cortex; SC, sleep control condition; SD, sleep debt condition.
doi:10.1371/journal.pone.0056578.g005

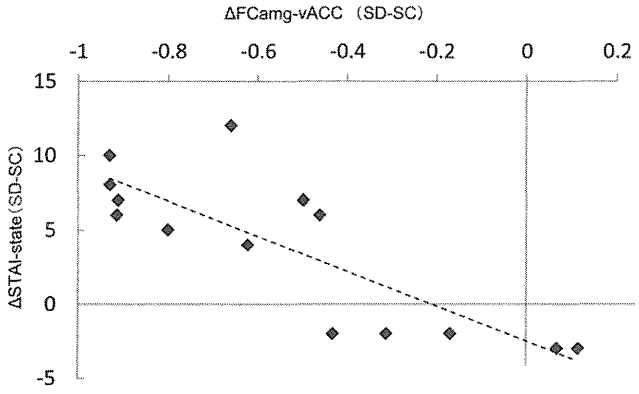

Figure 6. Correlation between the inter-session differences of amygdala–vACC functional connectivity and the inter-session differences STAI-state score. Inter-session differences between sleep control and sleep debt sessions of amygdala-vACC functional connectivity in response to fearful facial stimuli correlated negatively with inter-session differences of STAI-state score, $r(13) = .82$, $p = .0001$. Δvalue, inter-session difference between sleep control and sleep debt sessions for each value; vACC, ventral anterior cingulate cortex; SC, sleep control condition; SD, sleep debt condition; $FC_{amg\text{-}vACC}$, functional connectivity between amygdala and ventral ACC; STAI, State-Trait Anxiety Inventory.
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amygdala even in response to images associated with positive emotions [56]. It is reasonable to assume that this discrepancy is due to differences in the sleep conditions, such as total sleep deprivation in the former study and 5-day sleep restriction in the present one. It is possible that overnight sleep deprivation, more than accumulating sleep debt, affects the expression of positive emotion. In fact, overnight sleep deprivation elicits an antidepressant effect in patients with depression, as well as mood activation in healthy individuals [57,58,59,60,61]. Such effect and mood activation might be related to enhanced amygdala activation to positive emotional stimulus, as observed in a previous study. This notion is supported by the findings of a study in which antidepressant treatment enhanced amygdala activation in response to happy faces in patients with depression [62]. However, no similar phenomena have been reported in individuals with consecutive nights of sleep loss.

Unexpectedly, the presentation of emotional facial images under the non-conscious condition did not elicit changes in the amygdala activation after sleep restriction in the present study. A previous study showed that responses to masked priming are enhanced after a 2-day partial sleep deprivation [15]. Moreover, overnight total sleep deprivation strengthened the functional connectivity of amygdala with the sub-cortical region (midbrain) while diminishing the functional connectivity with the mPFC [14]. Based on these findings, we had expected to observe certain functional alteration in not only conscious processing through the cortical pathway, but also non-conscious processing through the subcortical pathway. However, no such alteration was observed in our participants. Any changes in non-conscious processing might be observable by adjusting the length of non-conscious image presentation, the intensity of the facial image stimuli, or increasing the sample size.

Interestingly, the decline in $FC_{amg\text{-}vACC}$ was correlated with the increase in %SWS and δ wave power in the early period of the sleep, but not with subjective sleepiness. Because the increase in SWS and δ wave power is thought to be a sensitive indicator of the sleep homeostatic process [63,64], it is possible that $FC_{amg\text{-}vACC}$ is

Table 4. Correlations between inter-session differences for fMRI data and questionnaire scores, and objective sleep debt Indices.

	ΔAmygdala activation	$\Delta FC_{amg\text{-}vACC}$
ΔSTAI-state	0.02	−0.82***
ΔPOMS Vigor	−0.22	0.49
ΔPOMS Depression	−0.20	−0.46
ΔPOMS Anger-Hostility	−0.38	0.11
ΔPOMS Fatigue	−0.27	−0.28
ΔPOMS Tension-Anxiety	0.13	−0.73**
ΔPOMS Confusion	0.02	−0.60*
ΔSSS	−0.41	0.01
$\Delta SWS_{2\ hrs}$	0.36	−0.59*
$\Delta Delta_{2\ hrs}$	0.03	−0.55*

Note. Δvalue, inter-session difference between sleep control and sleep debt sessions for each value; $FC_{amg\text{-}vACC}$, functional connectivity between amygdala and ventral ACC; SSS, Stanford Sleepiness Scale; STAI, State-Trait Anxiety Inventory; POMS, Profile of Mood States; $SWS_{2\ h}$, slow wave sleep of first 2 h from sleep onset; $Delta_{2\ h}$, delta wave power of first 2 h from sleep onset;
*$p<0.05$,
**$p<0.01$,
***$p<0.001$.
Degrees of freedom (df) = 13.
doi:10.1371/journal.pone.0056578.t004

associated with the neural basis that enhances homeostatic sleep pressure following short hours of sleep. It is also thought that the increase in homeostatic sleep pressure is caused by the accumulation of sleep promoting substances in brain, such as prostaglandin D2 and adenosine [65,66,67,68,69]. For example, adenosine receptor antagonists have been shown to reduce sleep time by increasing wake hours and reduce slow wave activity during sleep [65,66,68]. Recent studies have hypothesized that adenosine, accumulated in the synaptic cleft because of consecutive synaptic transmission, binds to the A1 receptor to suppress synaptic transmission [70]. It is possible that synaptic suppression by a sleep-promoting substance is involved in the attenuation of $FC_{amg\text{-}vACC}$ during sleep debt. Functional connectivity of the BOLD signal reportedly reflects a synaptic anatomical pathway [71]; therefore, it is expected that synaptic suppression by adenosine causes a decline in functional connectivity. This hypothesis is supported by a previous study which reported that a lack of sleep leads to attenuated resting state functional connectivity [72]. Moreover, a positron emission tomography (PET) study of human subjects revealed increased adenosine binding to the A1 receptor in regions including the vACC during sleep deprivation [73]. The use of PET with fMRI to elucidate the effects of sleep-promoting substances such as adenosine, including the effect of adenosine on the dynamics of neural activity in the brain, will likely elucidate the neural basis that alters the mechanism of emotion regulation.

Some limitations need to be taken into account when interpreting the present findings. First, participants in this study spent the first 3 days at home. Even though their sleep schedule was enforced by the use of the actigraph and mail alerts, their actual sleep time (8 h 5 min and 4 h 36 min in the SC and SD session, respectively) was longer than the scheduled sleep time (8 and 4 h in the SC and SD session, respectively). Despite the minor increase in sleep time, the SD session had a sleep loss of 3 h 30 min compared with the SC session. This level of sleep loss is rather frequently experienced in everyday life; however, the presence of sleep debt was confirmed on the last day of the session

by the increase in %SWS and δ wave power. Because sleep requirements vary among individuals, the same sleep schedule may result in large individual differences in sleep debt [74]. In other words, even if everyone were to be placed strictly on the same sleep schedule, the effects of sleep debt on brain activity and subjective moods would vary widely among individuals. If sleep can be restricted based on individual sleep requirements, functional changes in the amygdala and $FC_{amg\text{-}vACC}$ might be identified more accurately.

Conclusion

The results of this study indicate that a short-term sleep loss, which is often experienced in everyday life, can aggravate subjective mood including anxiety, and the mechanism appears to involve functional alteration of the amygdala and $FC_{amg\text{-}vACC}$. Long work hours, night-owl lifestyles, and an increase in shift work are the major contributors to sleep loss and thus the risk for depression [1,2,4,6,75,76,77,78]. Therefore, ensuring adequate sleep is an important lifestyle factor that deserves more attention in terms of managing mental health, including depression.

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Author Contributions

Conceived and designed the experiments: Y. Motomura SK KO YT Y. Moriguchi KM. Performed the experiments: Y. Motomura SK KO YT ME YK AH. Analyzed the data: Y. Motomura SK ME. Contributed reagents/materials/analysis tools: Y. Motomura SK KO YT ME Y. Moriguchi. Wrote the paper: Y. Motomura SK Y. Moriguchi SH KM.

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