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Effects of Auditory Environment on Freezing Behavior of Mice in a Novel Context

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ABSTRACT

Freezing behavior is a widely used parameter that represents the level of fear. A number of studies on emotional learning have used this behavior for quantification of fear that results from a cue or a context. Even though the expression of freezing behavior is based on the dynamic interaction of mice with the environment, the effect of environmental stimuli on freezing behavior has not been studied extensively because of its minority compared to the effect of conditioning-stimuli. In this study, we found that the auditory environment of a context affects the freezing behavior of a mouse in it. This effect was not observed when the mouse was exposed to the context for the first time. However, during the second exposure, the level of freezing behavior increased significantly in an intensity-dependent manner, while the type, pitch, and rhythm of additional sounds involved in the context did not induce notable effects. This intensity-dependent effect was unrelated to the level of fear and anxiety, reflecting another aspect of the freezing behavior as a parameter for recognizing the pattern of normal behaviors.

Key words: type of sound, pitch of sound, intensity of sound, rhythm of sound, freezing behavior, auditory environment

INTRODUCTION

Freezing behavior is a well-known response of an animal to an unavoidable fear stimuli (Brandão et al., 2007). Since it can be easily induced and discriminated, freezing behavior has been widely used as a parameter of fear expression in a number of studies (Lee et al., 2008). Fear conditioning, which includes both contextual and cued conditioning (Zhang et al., 2008; Inoue et al., 2009; Ko et al., 2009), exemplifies the experimental paradigm that uses freezing behavior for the measurement of fear level.

The freezing behavior is greatly affected by the stress and anxiety level of an animal. Since the environment modulates the basal level of stress and anxiety, it is one of the critical factors that affect freezing behavior. However, the effects of environmental stimuli on the freezing behavior have not attracted attention for a long time because they were considered as less important than that of conditioning stimuli. In this study, we tried to find out

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how the environment affects freezing behavior and what component of it is important for this effect.

We focused on the effect of the auditory environment. Many researchers have studied the influence of environmental sound on stress and anxiety levels. Noise from the traffic and industrial noise are known to increase the level of stress hormones, causing symptoms when chronically exposed to it (Passchier-Vermeer and Passchier, 2000). Similar results were revealed in animals. Noise from the housing environment has been reported to affect the condition of laboratory animals (Pfaff, 1974). While noise is known to have many negative effects, certain types of music are known to reduce the stress level. It has been reported that, listening to music during surgery can reduce the need for intraoperative sedatives (Ylmaz et al., 2003; Kang et al., 2008), and can help patients recover from health problems (Bradt and Dileo, 2009; Mantovan et al., 2009) or mental disorders (Gold et al., 2009).

In this present study, we demonstrated that environmental sound can also affect the freezing behavior of mice in a context. This effect was not observed while the mouse was exploring the context for the first time. However, during the second exploration, the freezing behavior of the mice within different auditory environment showed significant differences. Our data also show that the amplitude of this effect is dependent on the intensity of the sound, but not on the type, pitch or the rhythm of the sound.

MATERIALS AND METHODS

Subjects

9～11 week-old C57BL/6 male mice were purchased from the Orientbio Inc. for the experiments. All animals were housed under a 12 : 12 light cycle with food and water provided ad libitum. The temperature of the housing facility was kept at 23 degrees celsius. All works were conducted according to the policies and regulations for care and use of laboratory animals approved by Institutional Animal Care and Use Committee in Seoul National University.

Exposure to sound-containing contexts

Prior to the experiment, mice were handled in the holding room for four consecutive days. On the next day, mice were placed in a 40×40 cm white plastic box for the experiment. The background noise from the ventilation fan and from other unidentified sources was approximately 30 dB. Additional sounds were generated by a computer using a software (NHC tone generator 2.11), and turned on right before the entry of the mice. Seven different varieties of sound were used for the experiments: 5+4.69 kHz sine wave (5 kHz minor 2nd); 75 dB, 10+9.38 kHz sine wave (10 kHZ minor 2nd); 55 dB, 65 dB, and 75 dB, 15+14.07 kHz sine wave (15 kHz minor 2nd), 10+6.67 kHz sine wave (major 5th); 75 dB, white noise; 75 dB. Rhythms were also generated by a computer using a software (Hammerhead rhythm station 1.0). 400 beats per minute (bpm), 600 bpm, and 800 bpm of base-drum sound were generated for the experiment, and the intensity was adjusted to 55 dB. Turning on and off of the rhythms were controled as the other additional sounds. Mice were exposed to the context for 15 minutes each day for two consecutive days. The freezing behavior was recorded using video-based FreezeFrame fear-conditioning system and scored by Actimetrics Software.

RESULTS

Additional sounds presented within the auditory environment of a context affect the adaptation of mice to the context in a type-independent manner

In the first experiment, we examined whether the presentation of an additional sound could provoke different responses from mice in a context. We prepared a context and let mice explore it for fifteen minutes, with or without the presentation of an additional sound (75 dB). Mice were exposed to the same context for two days, and their behaviors were recorded and analyzed.

When mice were first exposed to the context with additional 75 dB of white noise, the level of freezing behavior was not significantly different from that of mice in the context with no additional sound (no sound, n=4, white noise, n=4, p=0.2,888; unpaired t test). However, during the second exposure performed on the following day, the freezing level in the white noise context was significantly higher than that in the no sound context (no sound, n=4, white noise, n=4, **p=0.0098; unpaired t test).

The same phenomena were also observed when we used different types of sound: minor 2nd (m2) dissonant harmony and major 5th (M5) consonant harmony, both of which were composed of two sine wave tones and had the 10 kHz tone as the upper tone. We could not see any difference in freezing behavior levels during the first exposure (no sound, n=4, minor 2nd, n=4, major 5th, n=6, white noise, n=4, p=0.7485; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 1A), while significant differences between sound-containing contexts and no sound context were observed on the second day (no sound, n=4, minor 2nd, n=4, major 5th, n=6, white noise, n=4, *p=0.0178; repeated one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 1B). Among the groups with any additional sound, there was no significant difference in freezing behavior level on both days.

Interestingly, there was a significant increase of freezing behavior on the second day in every group (no sound, n=4, **p=0.0088, minor 2nd, n=4, **p=0.0076, major 5th, n=6, ***p=0.0002, white noise, n=4, *p=0.0168; paired t test) (Fig. 1C). Although the extent of increase was much greater in sound-containing groups (no sound, n=4, minor 2nd, n=4, major 5th, n=6, white noise, n=4, *p=0.0260;

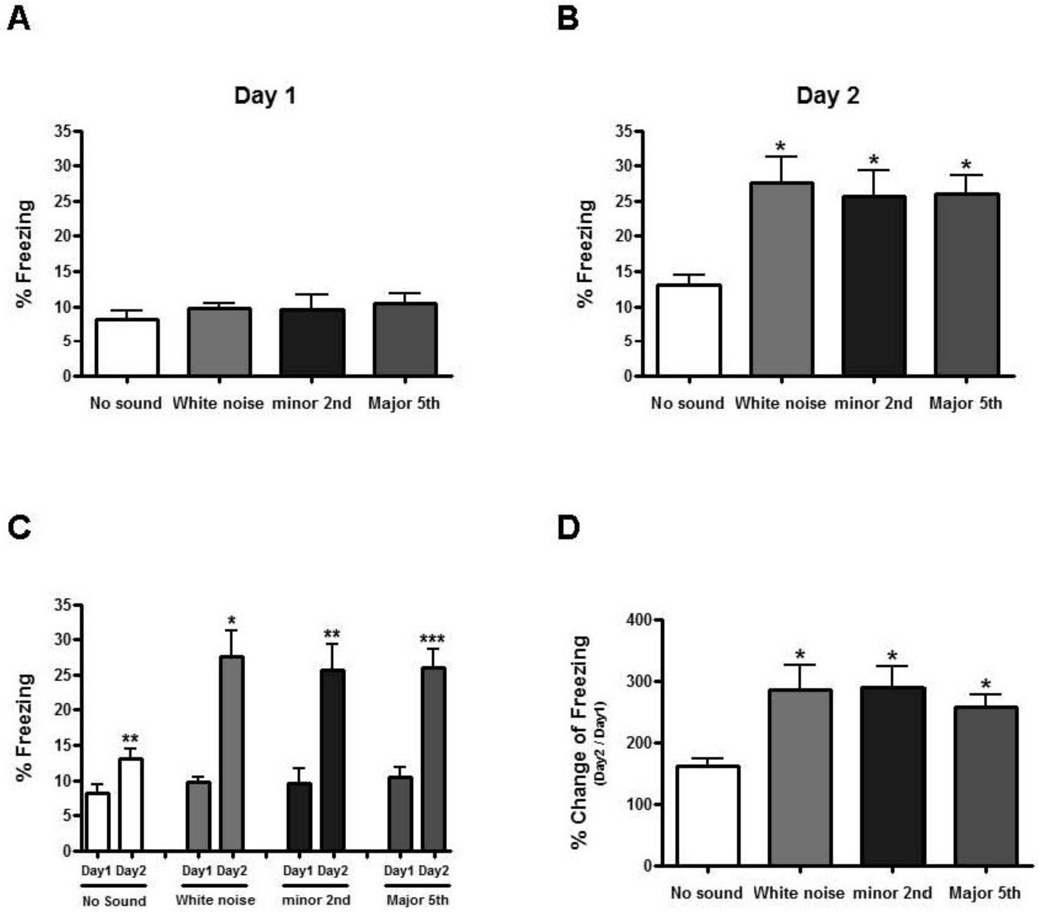

Fig. 1. Effect of different types of environmental sound on freezing behavior of mice. (A) Freezing behavior of the groups was similar on the first day ($p>0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (B) Freezing level in contexts with additional sounds became significantly higher than in no sound context on the second day (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (C) All groups showed tendency of significant increase (*$p<0.05$, **$p<0.01$, ***$p<0.001$; paired t-test between day 1 and day 2 of each group). Stars show the significance of the difference between two days of exposure of each group. (D) The amount of increase was higher in three sound-containing groups compared to the no sound group. However, no difference was observed among these three (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). No sound, n=4, minor 2nd, n=4, major 5th, n=6, white noise, n=4 in all panels. All data are expressed as a mean±the standard error of the mean (SEM). Error bars indicate SEM.

one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 1D), the increase was still significant in no sound group as well.

The pitch of the environmental sound did not affect adaptation of mice to the context

In the second experiment, we examined whether the pitch of the additional sound is an important factor that affects the behavior of mice in a context. The m2 dissonant harmony, which was used in the first experiment, was selected for the test. In one group, the pitch of the sound was raised to a higher frequency with 15 kHz sine wave as the upper tone, and in another group, it was adjusted to a lower frequency with 5 kHz sine wave as the upper tone. Again, all sounds were presented in the same intensity (75 dB).

As in the first experiment, there was no difference among groups on the first day (no sound, n=4, 5 kHz, n=4, 10 kHz, n=6, 15 kHz, n=4, p=0.7235; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 2A). However, the freezing level increased significantly on the second day (no sound, n=4, *p=0.0481, 5 kHz, n=4, *p=0.0274, 10 kHz, n=6, ***p=0.0004, 15 kHz, n=4, **p=0.0083; paired t-test) (Fig. 2C), and the extent of increase was significantly greater in the sound-containing groups compared to the no sound group (no sound, n=4, 5

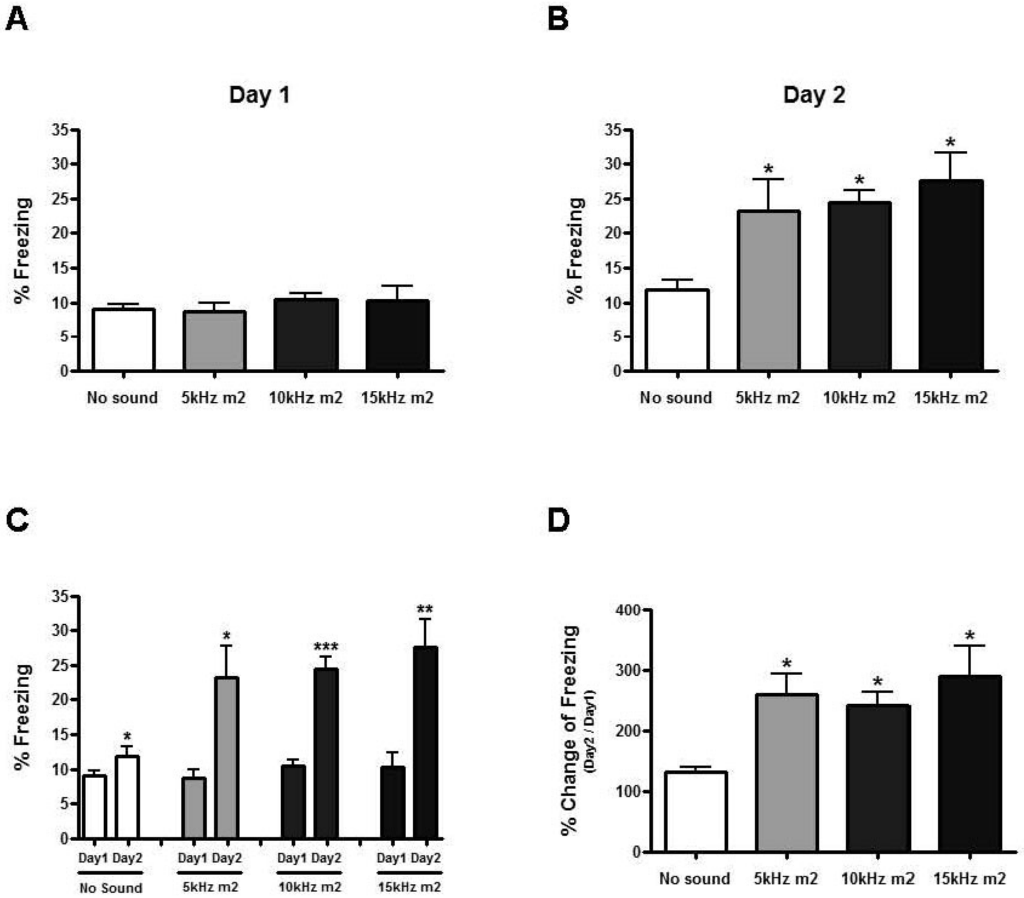

Fig. 2. Effect of the pitches of environmental sound on the freezing behavior of mice. (A) Freezing behavior of the groups was similar on the first day ($p>0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (B) Freezing level in contexts with additional sounds became significantly higher than in no sound context on the second day (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (C) All groups showed tendency of significant increase (*$p<0.05$, **$p<0.01$, ***$p<0.001$; paired t-test between day 1 and day 2 of each group). Stars show the significance of the difference between two days of exposure of each group. (D) The amount of increase was higher in three sound-containing groups compared to the no sound group. However, no difference was observed among these three (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). No sound, n=4, 5 kHz m2, n=4, 10 kHz m2, n=6, 15 kHz m2, n=4 in all panels. All data are expressed as a mean±SEM. Error bars indicate SEM.

kHz, n=4, 10 kHz, n=6, 15 kHz, n=4, *p=0.0222; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 2D). Again, no difference was observed among the groups with different types of sound (no sound, n=4, 5 kHz, n=4, 10 kHz, n=6, 15 kHz, n=4, *p=0.0163; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 2B).

Intensity of the environmental sound affected adaptation of mice to the context

In the third experiment, how the change in intensity of additional sound can affect adaptation of mice was examined. We chose dissonant harmony with 10 kHz sine wave as its upper tone, which was also used in previous experiments, and presented it in different intensities. 55 dB, 65 dB, and 75 dB were selected for the test.

On the first day, no significant difference was found among the groups (no sound, n=4, 55 dB, n=6, 65 dB, n=6, 75 dB, n=4, p=0.3047; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 3A). The tendency of the freezing behavior to increase on the second day was also observed in this experiment (no sound, n=4, *p=0.0104, 55 dB, n=6, **p=0.0094, 65 dB, n=6, **p=0.0013, 75 dB, n=4, ***p=0.0002; paired t-test) (Fig.

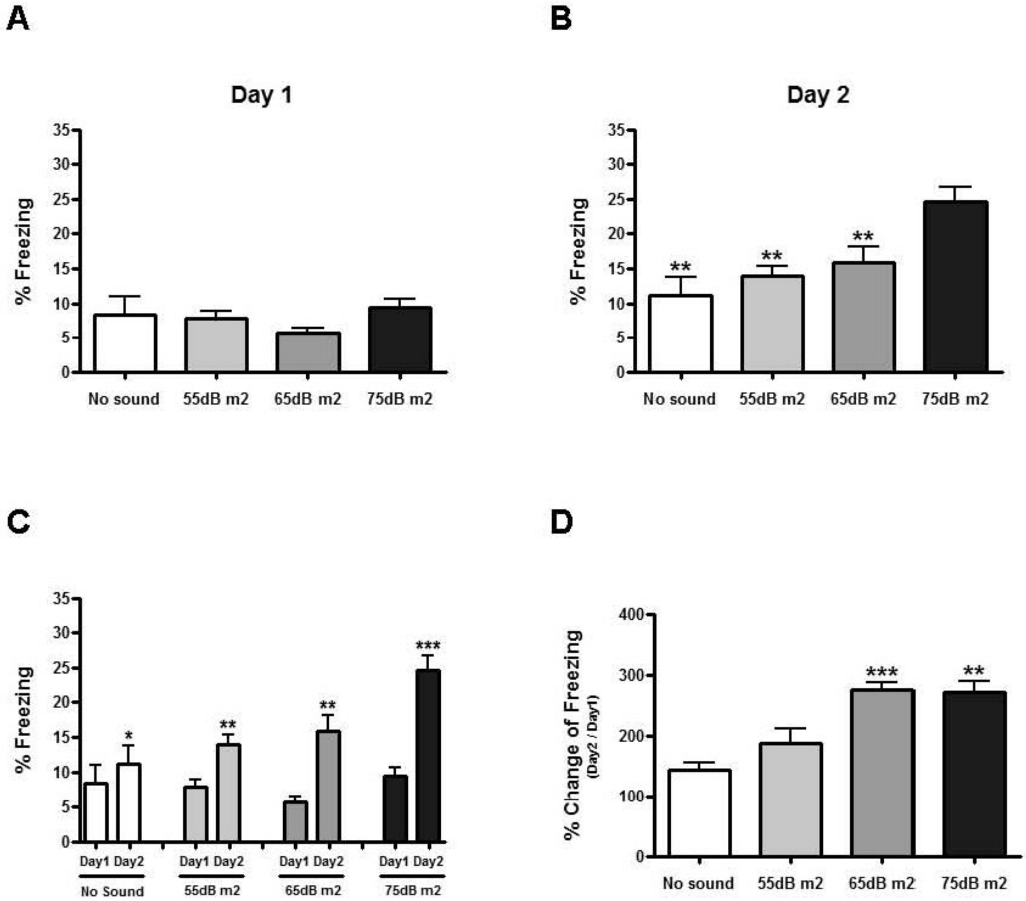

Fig. 3. Effect of the intensity of environmental sound on the freezing behavior of mice. (A) Freezing behavior of the groups was similar on the first day ($p>0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (B) Freezing level observed on the second day. m2 harmony of 75 dB group showed the greatest level of freezing (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (C) All groups showed tendency of significant increase (*$p<0.05$, **$p<0.01$, ***$p<0.001$; paired t-test between day 1 and day 2 of each group). However, the amount of increase was intensity-dependent. Stars show the significance of the difference between two days of exposure of each group. (D) The amount of increase was also intensity dependent. The freezing levels of 65 dB and 75 dB groups were significantly higher than those in the other two groups (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). No sound, n=4, 55 dB m2, n=6, 65 dB m2, n=6, 75 dB m2, n=4 in all panels. All data are expressed as a mean±SEM. Error bars indicate SEM.

3C). Even though the increase wes significant in all groups, the amount of increase was greatest in the group that was exposed to the context with 75 dB m2 harmony, and significantly lower in other groups (no sound, n=4, 55 dB, n=6, 65 dB, n=6, 75 dB, n=4, **p=0.0038; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 3B). Although the actual level of freezing behavior was lower than that of the 75 dB group, the amount of increase in the 65 dB group was also significantly higher than in other groups, suggesting the intensity-dependent manner of the freezing behavior change (no sound, n=4, 55 dB, n=6, 65 dB, n=6, 75 dB, n=4, **p= 0.0003; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 3D).

Rhythm of the environmental sound did not affect adaptation of mice to the context

In the next experiment, we tested the effect of the rhythm on the freezing behavior, another important feature of sound. Three kinds of rhythms, including 400 bpm, 600 bpm and 800 bpm, were selected for the experiment. The selection was made considering the heart beat of mice, which is known to be around 600 bpm (Halt et al., 2002). Beats were generated by computer with the intensity of 55 dB.

As in case of the m2 sound of 55 dB tested in the previous experiment, no significant difference was found among groups in both days of experi-

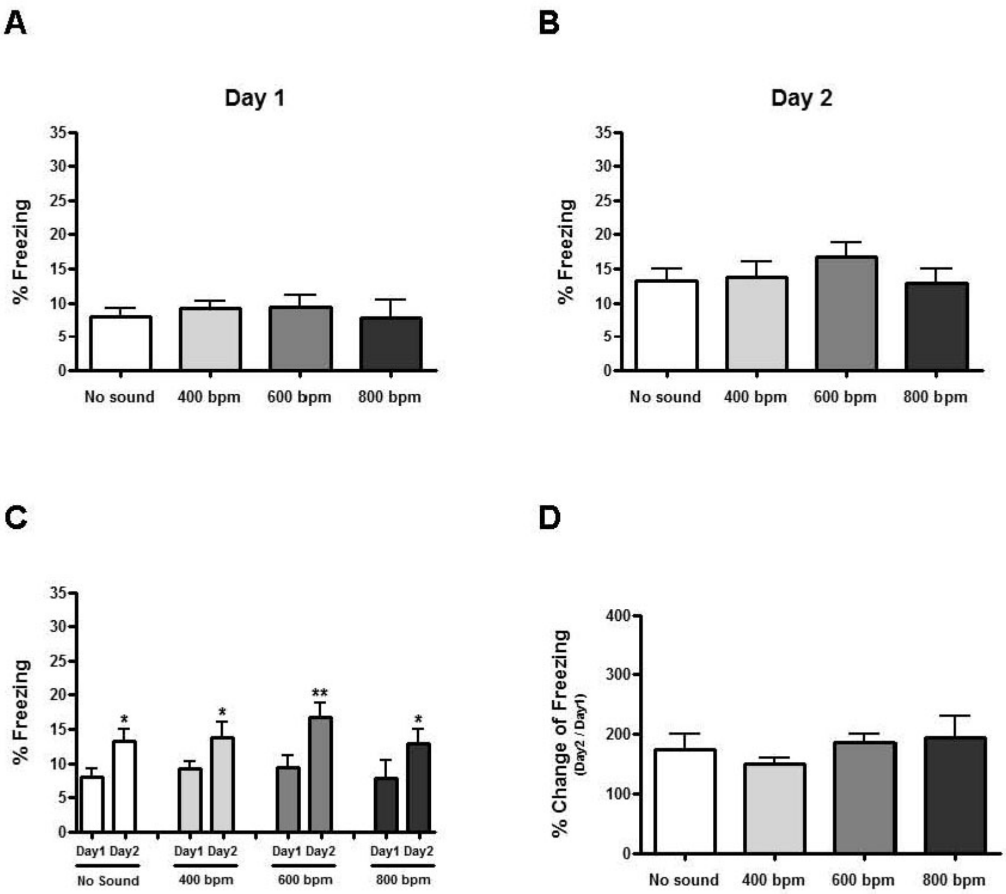

Fig. 4. Effect of the rhythm of environmental sound on the freezing behavior of mice. (A) Freezing behavior of the groups was similar on the first day ($p>0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (B) Freezing level observed on the second day. No significant difference was observed among groups ($p>0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (C) All groups showed tendency of significant increase (*$p<0.05$, **$p<0.01$; paired t test between day 1 and day 2 of each group). However, the amount of increase was intensity-dependent. Stars show the significance of the difference between two days of exposure of each group. (D) The amount of increase also showed no significant difference (*$p<0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). No sound, n=4, 55 dB m2, n=4, 65 dB m2, n=4, 75 dB m2, n=4 in all panels. All data are expressed as a mean±SEM. Error bars indicate SEM.

ment (no sound, n=4, 400 bpm, n=4, 600 bpm, n=4, 800 bpm, n=4 for both days, p=0.8937, for the first day, p=0.0555 for the second day; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 4A, B). However, the tendency of increase was also consistent, significant increase in the freezing level being observed in every group when we compared the levels of the second day with those of the first day (no sound, n=4, *p=0.0318, 400 bpm, n=4, *p=0.0337, 600 bpm, n=4, **p=0.0018, 800 bpm, n=4, *p=0.0342; paired t-test) (Fig. 4C). The extent of increase was also similar among the groups (no sound, n=4, 400 bpm, n=4, 600 bpm, n=4, 800 bpm, n=4, p=0.0984; one-way ANOVA and Newman-Keuls multiple comparison test) (Fig. 4D).

Difference in freezing behavior was not related to anxiety or locomotive activities

In the last experiment, we tested whether the existence of additional sound induces any increase of anxiety which can be a reason for the increased level of freezing behavior on the second day. We selected the m2 sound of 75 dB with 10 kHz sine wave as the upper tone, which showed stable effect in previous experiments for the open field test.

On the first day, the locomotive activity of mice, which is known to be associated with the level of anxiety, showed no difference between two groups (n=6 in both groups, p=0.7215; unpaired t-test) (Fig. 5A), showing that the level of anxiety was not significantly altered by the auditory environment.

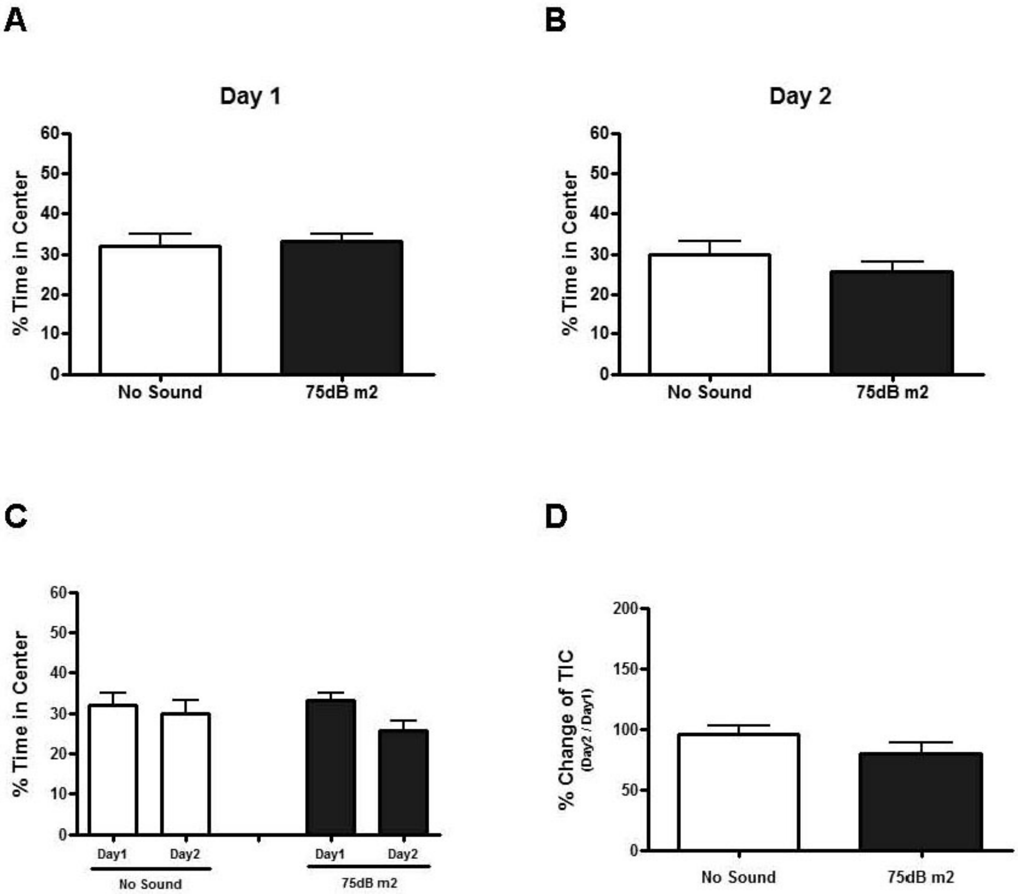

Fig. 5. Auditory environment-dependent alteration of freezing behavior is not related to the differential level of anxiety. (A) Comparison of the time spent in the central region (TIC) between two contexts. No significant difference was observed between contexts ($p>0.05$; unpaired t-test). (B) Comparison of the TIC on the second day. Again, the difference between two contexts was not significant ($p>0.05$; unpaired t-test). (C) The difference of TIC between Day 1 and Day 2 also was not significant in both contexts ($p>0.05$; unpaired t-test). (D) The amount of decrease was also similar between groups ($p>0.05$; unpaired t-test). No sound, n=6, 75 dB m2, n=6 in all panels. All data are expressed as a mean±SEM. Error bars indicate SEM.

This was also the case of the second day (n=6 in both groups, p=0.3088; unpaired t-test) (Fig. 5B). Interestingly, though previous results show that the level of freezing increases significantly during the second exposure, no significant decrease of time spent in the center area (TIC) was observed in both groups (no sound, n=6 in both days, p=0.4955, minor 2nd, n=6 in both days, p=0.0687; paired t-test) (Fig. 5C). The extent of TIC change was also similar between two groups (n=6 in both groups, p=0.2332; paired t-test) (Fig. 5D).

DISCUSSION

In the present study, we examined the effect of sound on the freezing behavior of mice in a context. Our data show that mice exposed to contexts with different auditory environments exhibit different levels of freezing behavior on the second day of exposure.

Considering that the existence of additional sounds is the only difference between the sound-containing context and the no sound context, it seems likely that the difference in the auditory environment is what affected the freezing behavior. If so, how is it affecting the alteration of the freezing behavior? The fact that the major difference is being observed on the second day implies that the experience made during the first exploration and the learning, or the remodeling of the nervous system induced by it, are critical factors for this behavioral alteration. However, it is difficult to predict actually how the learning induced in these two contexts were different from our results. We could not observe any difference in the freezing and anxiety level of mice on the first day. Since these two parameters cannot assess every aspect of mouse behavior, we cannot overlook the existence of undetected differences between mice in different environments. These undetected differences, which may be revealed through more extensive behavioral and molecular analysis, might have induced differential learning within a context during the first exposure, and resulted in the differential freezing behavior during the second exposure. Despite these open possibilities, our data show that freezing behavior is not a mere reflection of fear level, but it reflects a part of more complicated responses to a specific set of stimuli, which comprise a specific context.

It is very interesting that this freezing level increased significantly as a result of the learning, which occurred during the first day of context exposure. This tendency to increase was observed in all contexts we set up in this study, even though the extent of increase was dependent on the intensity of the sound. In a way, the increase in freezing behavior is similar to the decrease in exploratory behavior when faced with familiar objects, which was observed in the novel object recognition task (Lee et al., 2008). This suggests the possibility that this kind of change in behavior is an adaptive mechanism toward novel stimuli within the context. However, it requires more studies to figure out what is the meaning of decreased exploration behavior, and what is the actual system underlying it.

Taken together, our data show that when a mouse is exposed to a context and becomes adapted to it, the auditory environment of the context works as an important factor which affects the pattern of its behavior in the context, especially the freezing behavior. Mice showed increase in freezing behavior as a result of this adaptation, and the intensity of the sound involved in the context was the only factor that affected the extent of increase. Whether this phenomenon is only the property of the auditory environment, or is the common property of various sensational stimuli involved in the context, seems to be an interesting question. Though more studies are imperative for better characterization, we suggest that this study can be a good starting point for understanding the freezing behavior, as a parameter that reflects the status of live animals in which dynamic interactions with the environment are taking place.

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