Objective detection of microtremors in netrin-G2 knockout mice

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ABSTRACT

Background: Essential tremor is the most prevalent movement disorder and is thought to be caused by abnormalities in the cerebellar system; however, its underlying neural mechanism is poorly understood. In this study, we found that mice lacking netrin-G2, a cell adhesion molecule which is expressed in neural circuits related to the cerebellar system, exhibited a microtremor resembling an essential tremor. However, it was difficult to quantify microtremors in netrin-G2 KO mice.

New Method: We developed a new tremor detector which can quantify the intensity and frequency of a tremor. Using this system, we were able to characterize both the microtremors in netrin-G2 KO mice and low-dose harmaline-induced tremors which, to date, had been difficult to detect. Alcohol and anti-tremor drugs, which are effective in decreasing the symptoms of essential tremor in patients, were examined in netrin-G2 KO mice. We found that some drugs lowered the tremor frequency, but had little effect on tremor intensity. Forced swim as a stress stimulus in netrin-G2 KO mice dramatically enhanced tremor symptoms.

Comparison with Existing Methods: The detection performance even for tremors induced by low-dose harmaline was similar to that in previous studies or more sensitive than the others.

Conclusions: Microtremors in netrin-G2 KO mice are reliably and quantitatively detected by our new tremor detection system. We found different effects of medicines and factors between human essential tremors and microtremors in netrin-G2 KO mice, suggesting that the causations, mechanisms, and symptoms of tremors vary and are heterogeneous, and the objective analyses are required.

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1. Introduction

Essential tremor (ET) is the most prevalent movement disorder (Louis, 2005). To understand the neural mechanisms of ET, an animal model of ET using harmaline, a tremorgenic drug, has been used conventionally (Wilms et al., 1999). Harmaline-induced tremors demonstrate 8–16 Hz trembling in rodents and are diminished not only by therapeutic medicines, such as propranolol and primidone, but also by alcohol ingestion, which is common in human ET (Martin et al., 2005). Harmaline enhances synchronized firing of the inferior-olivary neurons (Llinas and Yarom, 1986) and synchronous inputs to the cerebellar Purkinje cells, which may be the core mechanism of tremor generation (Handforth, 2012). Recently, genetic technologies have advanced, and various mutant and transgenic animals demonstrating tremors and/or ataxia have been generated. These animals could be suitable probes to address the neural mechanisms of ET (Handforth, 2016).

Netrin-G2 is a presynaptic cell adhesion molecule which belongs to the netrin family, which are extracellular proteins which guide cellular and axonal migrations in vertebrate brains. In the cerebellum, netrin-G2 is expressed in Bergmann glia of the cortex and the deep cerebellar nucleus and in the inferior olivary nucleus in the medulla oblongata (Yaguchi et al., 2014). Netrin-G2 KO mice show various phenotypes, including functional deficits in motor control (Matsukawa et al., 2014; Zhang et al., 2016; Zhang W. et al., 2008). It is notable that the netrin-G2 KO mice showed microtremors in action; however, the microtremors were detectable qualitatively by an expert among the experimenters.

Quantitative methods of tremor detection for rodent models have...
been developed since the 1950s based on various tools including: wire coils and magnets (Dill et al., 1968; Moore et al., 1957; Shinozaki, 1984), gramophone or phonograph pick-ups (Agarwal and Bose, 1967; Ahmed and Taylor, 1959; Kelly and Naylor, 1974), strain gauges (Bekar et al., 2008; Fowler et al., 2001; Gerhart et al., 1982; Johnson et al., 1986; Martin et al., 2005; Yen and Day, 1965), and electromyography (Bekar et al., 2008; Günther et al., 1983; Hosoi et al., 2013; Milner et al., 1995; Sinton et al., 1989; Yamazaki et al., 1979). Other methods include piezo devices (Da Fonseca et al., 2001; Oris et al., 1969; Paterson et al., 2009; White et al., 2016), speakers (Gothoni et al., 1981; Kralic et al., 2005; Remington and Anisman, 1976), accelerometers (Hallberg et al., 1985; Kistler et al., 2002; Milner et al., 1995; Park et al., 2011; Sinton et al., 1989), ultrasonic transducers (Young et al., 1996, 2000), a Hall effect sensor (Hutchinson et al., 2007), an optical sensor (Cavallio et al., 2008), video cameras (Baker et al., 2000; Finn et al., 1997), and a smartphone (Carlson et al., 2019). Some of them have been commercialized (Fowler et al., 2001; Hutchinson et al., 2007; Martin et al., 2005); however, there is no standard method. Most of these methods are able to detect prominent tremors, such as harmaline-induced tremors, and there are no devices to detect and quantitatively assess weak tremors, such as microtremors in netrin-G2 KO mice. In this study, we developed a new system for detecting and evaluating weak tremors and the system to investigate microtremors in netrin-G2 KO mice.

2. Materials and methods

2.1. Animals

Netrin-G2 (Ntn2g) KO mice were generated previously and maintained as C57BL/6J congenic heterozygotes (Nishimura-Akiyoshi et al., 2007). PCR genotyping was conducted using primers as follows: primer-a (5′-CTCTTCAATGGAGACCCAAG-3′), primer-b (5′-TGAAGATAAAGGGATGAGG-C3′), and primer-c (5′-GGAGGTAACTTGGAGATAC-3′). PCR products of 322 bp and 166 bp were amplified using primer-a/primer-b and primer-a/primer-c from the wild-type and Ntn-g2 KO alleles, respectively. Mice of both sexes were used at the age of 3–100 weeks. Ntn2g-LacZ-KI mice (8 weeks old) used for X-gal staining were reported previously (Yaguchi et al., 2014). Images of brain sections were captured at 200× magnification using a slide scanner (NanoZoomer RS, Hamamatsu Photonics). All experimental procedures were performed in accordance with the guidelines of the RIKEN Institutional Animal Care and Experimentation Committee.

2.2. Tremor detection system

Our developed tremor detector was composed of a cubic acrylic chamber with a side length of 15 cm, a polystyrene base plate, four piezo films (LTDO-028K, Measurement Specialties, Inc.) which were inserted through slits on each side of the chamber, a microcontroller (Gainer mini, RT Corporation), and a computer (Fig. 2A). The piezo films supported the base plate from the bottom and sensed vibrations ascribed to the mouse’s movement when a mouse was placed on the base plate. Its voltage signals were converted into digital signals at an 8-bit depth via the microcontroller and recorded to a storage drive in a computer at a 60-Hz sampling frequency, using the original software written in Processing (https://processing.org). Using the detector, we recorded each mouse’s motion for 5 min individually while the mouse explored the environment in the chamber. The behavior of the mouse was simultaneously monitored by a web camera located above the chamber during the tremor recording (Fig. 2B).

2.3. Tremor detection algorithm

We established a novel algorithm to detect weak tremors in mice using the new tremor detector. The tremor detection algorithm was constructed using the following procedures (Fig. 4G): (Step 1) the mouse’s motion signals from each piezo detector were converted into a power spectrum with a fast Fourier transform, where the spectra had a frequency ranging from 0 to 30 Hz; (Step 2) the power spectra which were converted were transformed into logarithms; (Step 3) linear functions fitted to the logarithmic power spectra were subtracted from the spectra itself; (Step 4) the peak frequencies of the power spectra were linearly fitted where specified, in the range of 5–25 Hz, after the spectra were smoothed to where the peak was defined as the mean of the frequencies at the top 10 % of the power values; (Step 5) the tremor index was calculated as described below:

$$T_l = \frac{\sum P_{peak} - \sum P_{ref}}{\sum P_{peak} + \sum P_{ref}}.$$ 

where $T_l$ represents the tremor index, $\sum P_{peak}$ is the integration of powers at a peak frequency of ±1.5 Hz, and $\sum P_{ref}$ is the reference integrated power ranging from 27 to 30 Hz. Signals with peak frequencies less than 7 Hz or greater than 20 Hz were excluded from the analysis as outliers. All the processes were computed using MATLAB (MathWorks, Inc.).

2.4. Motion detection algorithm

To evaluate whether mouse motion can actually be identified from the sensor data, we compared the sensor data with the video data by calculating correlation coefficient (CC). The mouse’s silhouette was extracted from each movie frame using morphological processes (bin- alization and dilation-erosion) and its centroid was determined (Fig. S1A). Mouse motion traces derived from the video data and sensor data were defined as the variation of the distance of the mouse’s centroid between adjacent frames and an average waveform of the four sensors, respectively (Fig. S1B). Both motion traces were resampled at 60 Hz and smoothed by a moving average filter (filter size: 2 s). The active period was obtained by thresholding the motion traces from the sensor data.

Bearing behaviors were detected manually from the video data. All the processes were computed using MATLAB (MathWorks, Inc.).

2.5. Drug administration

Harmaline (H1392, Sigma) dissolved in saline was injected intra-peritoneally (i.p.). Doses of 3.7, 5, 10, and 20 mg/kg were administered. Tremors usually occurred 10–20 min after harmaline administration. Ethanol (20 % w/v, 4 g/kg in saline) (Martin et al., 2005) or water was administered with an oral gavage tube. Propranolol (10 mg/kg in PBS, P0884, Sigma) (Paterson et al., 2009), zonisamide (50 mg/kg in DMSO, 2625, Tocris Bioscience) (Miwa et al., 2011), and MK-801 (50 mg/kg in PBS, 0995, Tocris Bioscience) (Kralic et al., 2005) were injected i.p. Saline was used as the vehicle control. Tremor was measured 30 min after drug administration.

2.6. Forced swim test and measurement of body temperature

Mice were placed in a beaker (5 L, 18 cm, diameter) filled halfway with water at 24 °C. To measure their body temperature, a microchip transponder (IPTT-300, BMDS, Inc.) was subcutaneously implanted into their back and their body temperature was captured by near-field communication using a handheld scanner (DAS-7007S, BMDS, Inc.) just before the swim and every minute for 10 min after the swim. Their wet hair was gently wiped after the swim.

2.7. Statistical analysis

The data were analyzed using a Student’s t-test, Mann-Whitney U test, and a Kruskal-Wallis test. The Steel-Dwass test or Mann-Whitney U test with a Bonferroni correction was used for post-hoc multiple comparisons. All values are represented as means ± SEM. The analysis was
conducted in MATLAB (MathWorks, Inc.) or R (R core team).

3. Results

3.1. Localization of netrin-G2 expression in the brain

Netrin-G2 is expressed in various brain areas. To demonstrate its localization, we performed X-gal staining of Ntng2-LacZ-KI mouse brains at 8 weeks of age (see Materials and methods). Netrin-G2 was expressed in layer II/III, IV, and VI neurons in the cerebral cortex, dentate granule cells, and hippocampal pyramidal neurons (Fig. 1A and B). Netrin-G2 was also localized in the basolateral amygdala nucleus (Fig. 1C). In the cerebellum, netrin-G2 was expressed markedly in the deep cerebellar nucleus (DCN) and modestly in the Bergmann glia (Fig. 1C and D). Netrin-G2 appeared in the inferior olivary nucleus (IO) in the medulla oblongata (Fig. 1C). These results are consistent with previous reports (He et al., 2018; Nishimura-Akiyoshi et al., 2007; Yaguchi et al., 2014).

3.2. Development of the microtremor detection system

Netrin-G2 KO mice were generated (Fig. 1E, see Materials and methods). We found microtremors as a phenotype of netrin-G2 KO mice. The microtremors could be visually detected by an expert. To detect the microtremors objectively, we developed a new tremor detection system for small animals (Fig. 2, see Materials and methods). The mice could move freely on a polyurethane base plate in an acrylic chamber (15 × 15 × 15 cm) of the system. All of the mouse movements, including the tremors, were transduced into voltage changes using piezoelectric film sensors attached to the bottom of the base plate. The 10-s motion traces are depicted in Fig. 3A. Movements accompanied by the behavior were detected in both genotypes, and notably, vibrations with small amplitudes were observed in the KO mice. Harmaline-induced tremors prominently occurred in both genotypes (Fig. 3C and Video 1) and were clearly identified in the 10–15-Hz frequency band of the spectrograms (Fig. 3D). However, microtremors in the KO mice were not obvious in the spectrogram (Fig. 3B).

To quantify the microtremors, we designed a novel algorithm as follows: the movement time courses were transformed into power spectra (Fig. 4A and D); then, a logarithmic transform of the frequency spectra and a linear fitting and subtraction on the semi-log plots were conducted (Fig. 4B and E). Peak detection of the processed spectra and calculations of the mean power in the peak and reference frequency bands were performed (Fig. 4C and F), and the tremor index was defined as the power ratio of the peak and reference frequency bands (Fig. 4G, see Materials and methods). Simultaneously, the frequency peaks were detected using this algorithm in both non-treated and harmaline-treated KO mice (Fig. 4C and F).

3.3. Validation of the microtremor detection system

To evaluate tremor detection using our system, we applied it to a low dose of harmaline. In wild-type (WT) mice, the tremor index at a dose of 5 mg/kg was significantly higher than that observed in the controls (Fig. 5A, $\chi^2(3, 31) = 25.9, p = 3.26 \times 10^{-5}$, Kruskal-Wallis test; control [n = 6]; 3.7 mg/kg [n = 7], p = 0.628; 5 mg/kg [n = 7], p = 0.0221; 10 mg/kg [n = 5], p = 0.00433; 20 mg/kg [n = 11], p = 1.62 × 10^{-4}, Mann-Whitney U test). The tremor frequencies at a 5.0 mg/kg dose did not differ from each other (Fig. 5B, $\chi^2(2, 20) = 1.52, p = 0.468$, Kruskal-Wallis test). The tremor frequency was not applicable (N/A) if the tremor index was comparable to that of the control (Fig. 5A and B). We applied the system to measure microtremors in netrin-G2 KO mice and found a significant difference in the tremor indices between WT and KO mice. (Fig. 5C, WT [N = 69, n = 20], KO [N = 66, n = 16], p = 9.34 × 10^{-14}, Mann-Whitney U test, where N is the number of sessions and n is the number of mice). Harmaline produced strong tremors in the KO mice as well as in WT mice (Fig. 5D, control [N = 66, n = 20], harmaline [N = 6, n = 4], p = 5.72 × 10^{-5}, Mann-Whitney U test). The tremor frequency in the harmaline-treated KO mice was slightly lower than that in the KO mice without harmaline (Fig. 5E, p = 0.0119, Mann-Whitney U test).

The similarity between the motion traces from the sensor data and video data was confirmed by the CC (Fig. S1B, see Materials and methods), and the average value was 0.620 (SEM = 0.0147, n = 40 [WT, n = 20; KO, n = 20]). This implied that the two traces were reasonably similar. The tremor indices did not differ between the whole and active periods; however the indices dropped significantly during the resting period in both genotypes (Fig. S1C, WT [n = 20], KO [n = 20], $\chi^2(5, 114) = 80.2, p = 7.52 \times 10^{-16}$, Kruskal-Wallis test; WT [whole] vs. WT [active], t = 1.68, p = 0.547; WT [active] vs. WT [resting], t = 4.87, p = 1.66 × 10^{-5}; KO [whole] vs. KO [active], t = 1.60, p = 0.601; KO [active] vs. KO [resting], t = 4.95, p = 1.10 × 10^{-5}; WT [whole] vs. KO [whole], t = 3.57, p = 0.00480; WT [active] vs. KO [active], t = 3.73, p...
This indicated that tremor did not occur during the resting period. The tremor frequencies were comparable between the whole and active periods in the KO mice (Fig. S1D, \( p = 0.695 \), Mann-Whitney U test). The ratio of the active period to the whole period (active ratio) was larger in the KO mice than in WT mice (Fig. S1E, \( p = 0.00905 \), Mann-Whitney U test). To verify whether the length of the active period had any influence on the tremor index, we analyzed the tremor characteristics of the KO mice by dividing them into two groups, the top half and the bottom half of the active ratio (Fig. S1F, top \([n = 10]\), bottom \([n = 10]\), \( p = 1.83 \times 10^{-4} \), Mann-Whitney U test). The results demonstrated that neither the tremor indices nor the tremor frequencies changed between the two groups (Fig. S1G and H, tremor index, \( p = 0.241 \), Mann-Whitney U test). This indicates that the length of the active period does not affect the tremor characteristics.

We focused on rearing behavior as a specific type of mouse behavior and manually detected the rearing behaviors from the video data. The tremor indices decreased significantly during rearing behaviors compared to the active period (Fig. S1I, WT \([n = 16]\), KO \([n = 14]\), \( \chi^2(3, 56) = 37.1, p = 4.34 \times 10^{-8} \), Kruskal-Wallis test; WT [active] vs. WT [rearing], \( t = 3.84, p = 6.98 \times 10^{-4} \); WT [active] vs. KO [active], \( t = 3.78, p = 8.92 \times 10^{-4} \); KO [active] vs. KO [rearing], \( t = 4.00, p = 3.73 \times 10^{-4} \), multiple comparisons with the Steel-Dwass test). The ratio of the rearing period, during which rearing behaviors were observed, to the whole period (rearing ratio) was significantly larger in the KO mice than in WT mice (Fig. S1J, \( p = 0.0261 \), Mann-Whitney U test). There was no difference between WT and KO mice in the percentage of overlap with the rearing period during the active period (Fig. S1K, \( p = 0.851 \), Mann-Whitney U test). These results indicate that activity, including rearing behaviors, is increased in the KO mice; however, microtremors are weakened during rearing behaviors. The increased activity in netrin-G2 KO mice is consistent with a previous study (Zhang et al., 2016).

The tremor indices of both genotypes were independent of age and body weight (Fig. 5F and G, WT \([N = 69, n = 20]\), KO \([N = 66, n = 16]\), age vs. tremor index: WT [solid line], \( R^2 = 0.00160, p = 0.744 \); KO [dotted line], \( R^2 = 0.00559, p = 0.551 \); body weight vs. tremor index:
There was no difference in the age-dependency between the body weights in WT and KO mice (Fig. S2A, age vs. body weight: WT [solid line], $R^2 = 0.186$, $p = 2.16 \times 10^{-4}$; KO [dotted line], $R^2 = 0.163$, $p = 7.85 \times 10^{-4}$, Student’s t-test), and the mean body weights of both genotypes (Fig. S2B, WT [N = 69, n = 20], KO [N = 66, n = 16], $p = 0.258$, Mann-Whitney U test). These results demonstrated that this tremor detection system could quantify tremor amplitudes with a wide dynamic range, including between microtremors of netrin-G2 KO mice and harmaline-induced tremors, for any

**Fig. 4.** Tremor detection algorithm. (A) Examples of the power spectra corresponding to the data, as in Fig. 3 (step 1). (B) Logarithmic transformations of the power spectra and the linear fitting (steps 2 and 3). Solid lines in red indicate the linear fit lines. Dashed lines are the baselines after the subtraction of the fit lines. (C) Peak-frequency detection and calculation of the tremor index (steps 4 and 5). Yellow lines show the smoothed curves of the power spectra processed by step 3. White vertical lines in the left panels exhibit the peak frequencies. Magenta and cyan hatches indicate the ranges, including the frequency peaks and the reference range, respectively. Magenta and cyan lines in the middle and left panels show the average power at each range as mentioned above. (D–F) Example data with harmaline administration following steps 1–5 as in (A–C). (G) Steps of the tremor detection process.

**Fig. 5.** Tremors induced by harmaline and knocking out netrin-G2. (A) Harmaline dose-dependency on tremor index in wild-type mice. Significance is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001. (B) Tremor frequency shows no harmaline dose-dependency in wild-type mice. (C) Tremor index in drug-naive wild-type (WT) and netrin-G2 KO mice. (D) Harmaline-induced tremors in netrin-G2 KO mice are significantly larger than those in the controls. (E) Tremor frequency value is slightly decreased by the harmaline treatment in netrin-G2 KO mice. (F and G) Tremor indices in both genotypes have no dependencies on age or body weight. Closed and open bars/dots represent WT and KO, respectively.

**Fig. 6.** Tremor characteristics of netrin-G2 KO mice under the alcohol intake and forced swim conditions. (A) No effects on the tremor index are seen under alcohol intake for wild-type (WT) and netrin-G2 KO mice. Significance is indicated by: **p < 0.01. (B) Significant decrease (**p < 0.001) of the tremor frequency value following alcohol intake in the KO mice. (C) There is no difference in the tremor indices before and after the forced swim in WT mice, whereas severe tremors are induced in the KO mice after the swim. (D) Tremor frequency value decreases in the KO mice after the swim. Closed and open bars represent WT and KO, respectively.
weight and age range.

### 3.4. Influence of alcohol intake and stress on tremors in netrin-G2 KO mice

Alcohol intake is known to temporarily ameliorate tremors in patients (Mostile and Jankovic, 2010; Rajput and Rajput, 2014). In contrast, mental and physical stress can enhance tremors (Handforth and Parker, 2010). Alcohol (ethanol) or water was transgastrically administered to both the KO and WT mice. The tremor indices showed no significant difference between alcohol and water administrations in either genotype (Fig. 6A, WT [n = 14], KO [n = 11], χ²(3, 46) = 16.2, p = 0.00103, Kruskal-Wallis test; WT [water] vs. WT [alcohol], t = 2.07, p = 0.164; WT [water] vs. KO [water], t = 3.34, p = 0.00466; WT [water] vs. KO [alcohol], t = 3.01, p = 0.0139; WT [alcohol] vs. KO [alcohol], t = 1.20, p = 0.624; KO [water] vs. KO [alcohol], t = 1.41, p = 0.491, multiple comparisons with the Steel-Dwass test). In the KO mice, the tremor frequency in alcohol-intake mice was lower than that in the water-intake mice (Fig. 6B, p = 1.07 × 10⁻⁴, Mann-Whitney U test). These results demonstrate that alcohol has no effect on the tremor index in netrin-G2 KO mice but shifted the tremor frequency to a lower value, suggesting that alcohol mechanistically influences microtremors.

Next, mice were forced to swim in 24 °C water for 10 min in order to examine the effects of stress, and tremors were measured before and after the swim. There was an obvious increase in the tremor index of the KO mice after the swim, but not in that of WT mice (Fig. 6C and Video 2, WT [n = 11], KO [n = 12], χ²(3, 42) = 32.1, p = 4.87 × 10⁻⁷, Kruskal-Wallis test; WT [before swim] vs. WT [after swim], t = 9.52, p = 0.777; WT [before swim] vs. KO [before swim], t = 3.26, p = 0.00609; WT [after swim] vs. KO [after swim], t = 4.06, p = 2.84 × 10⁻⁴, KO [before swim] vs. KO [after swim], t = 4.16, p = 1.89 × 10⁻⁴, multiple comparison with the Steel-Dwass test). Additionally, the tremor frequency value significantly decreased after the swim in the KO mice (Fig. 6D, p = 7.31 × 10⁻⁴, Mann-Whitney U test). The body temperatures after the swim in cold water dropped down in both genotypes but showed no significant differences between the mice of either genotype (Fig. S3, WT [N = 9, n = 7], KO [N = 11, n = 7], Mann-Whitney U test). These results indicate that the forced swim did not induce physiological shivering in WT mice but did induce severe tremors in netrin-G2 KO mice.

### 3.5. Anti-tremor-drug effects on tremors in netrin-G2 KO mice

To investigate whether anti-tremor drugs improve microtremors in netrin-G2 KO mice, we administered propranolol (β-adrenergic blocker), zonisamide (anticonvulsant, T-type calcium channel blocker), and MK-801 (non-competitive N-methyl-D-aspartate [NMDA] glutamate receptor blocker). Propranolol and zonisamide did not affect the tremor index, whereas MK-801 slightly reduced the tremor levels (Fig. 7A, control [n = 32], propranolol [n = 7], zonisamide [n = 7], MK-801 [n = 7], χ²(3, 49) = 9.23, p = 0.0264; Kruskal-Wallis test; control vs. propra., t = 0.366, p = 0.983; control vs. zonis., t = 0.135, p = 0.528; control vs. MK-801, t = 2.74, p = 0.0308, multiple comparisons with the Steel-Dwass test). Propranolol and MK-801 had no significant effect on tremor frequency compared to the control, whereas zonisamide significantly decreased the tremor frequency (Fig. 7B, χ²(3, 49) = 17.2, p = 6.33 × 10⁻⁴; Kruskal-Wallis test; control vs. propra., t = 0.0366, p = 0.999; control vs. zonis., t = 4.06, p = 2.83 × 10⁻⁴; control vs. MK-801, t = 0.10, p = 0.999, multiple comparison with the Steel-Dwass test). The active ratio of MK-801 treatment in the KO mice significantly decreased (Fig. 7C, χ²(3, 49) = 12.8, p = 0.00509; Kruskal-Wallis test; control vs. propra., t = 1.32, p = 0.552; control vs. zonis., t = 2.56, p = 0.0510; control vs. MK-801, t = 2.78, p = 0.0277, multiple comparisons with the Steel-Dwass test). This showed the influence of immobilization by MK-801.

In addition, to verify whether the effect of MK-801 on microtremors was owing to immobilization, we examined the tremor characteristics during the active period (Fig. 7D and E, tremor index: χ²(3, 49) = 11.9, p = 0.00784, Kruskal-Wallis test; control vs. propra., t = 0.329, p = 0.988;
control vs. zonis., $t = 1.90, p = 0.227$; control vs. MK-801, $t = 2.89, p = 0.0201$; tremor frequency: $\chi^2(3, 49) = 17.6, p = 5.25 \times 10^{-4}$, Kruskal-Wallis test; control vs. propra., $t = 1.35, p = 0.528$; control vs. zonis., $t = 3.73, p = 0.00109$; control vs. MK-801, $t = 1.32, p = 0.552$, multiple comparisons with the Steel-Dwass test). Similar to the results of the whole period, the tremor index was reduced by MK-801 treatment in the active period. This indicates that the effect of MK-801 on microtremors cannot be explained solely by the influence of immobilization. These results suggest that microtremors in netrin-G2 KO mice are not exactly the same as human ET; however, MK-801 did reduce microtremors.

4. Discussion

We quantitatively detected the microtremors observed in netrin-G2 KO mice using our newly developed tremor detection system. The system was able to detect tremors in a wide range of amplitudes, from the microtremors in netrin-G2 KO mice to severe harmaline-induced tremors. Using the detection system, we examined the effects of alcohol intake and forced swims, a reducer and an enhancer of human ET, respectively, on the microtremors in netrin-G2 KO mice. We demonstrated that the effect of alcohol was limited, but forced swims enlarged the microtremor amplitudes. Furthermore, we employed pharmacological approaches to investigate the resemblance of tremorgenesis in netrin-G2 KO mice to human ET and found that the effect of anti-tremor drugs was limited, but some of them changed the tremor characteristics.

4.1. Comparative evaluation of tremor detectors

The usual dose of harmaline for mice is 10–20 mg/kg i.p. (Agarwal and Bose, 1967; Ahmed and Taylor, 1959; Bekar et al., 2008; Fowler et al., 2001; Milner et al., 1995; Park et al., 2010), which produces an obvious tremor in the range of 11–16 Hz. Our system was sufficient to detect tremors in this range (Fig. 5A). Detection performance even at lower doses (5 mg/kg) was similar to previous studies (Amrutkar et al., 2020; Hutchinson et al., 2007) or more sensitive than others (Da Fonseca et al., 2001; Martin et al., 2005; Paterson et al., 2009), except for one study, which detected tremors in task-dependent voluntary forelimb movements under restraint (Stanford and Fowler, 1998). Therefore, our system is the most sensitive for performing harmaline-induced tremor detection in freely moving mice. This high sensitivity allowed us to detect microtremors in netrin-G2 KO mice (Fig. 5C).

In this system, an ultra-lightweight polystyrene stage plate is directly supported by four piezoelectric films in order to improve the conduction efficiency of the mechanical vibrations. The elasticity of the film itself provides a high dynamic range and high resistance to the weight of the mice (Fig. 5G). Although the data were recorded at a relatively low sampling frequency to avoid the influence of humming noise in this study, further improvement in the signal-to-noise ratio can be achieved by eliminating common mode noise using an instrumentation amplifier, which will allow the examination of the characteristics of higher frequency bands.

4.2. Possible neural circuits involved in microtremors in netrin-G2 KO mice

ET expression is thought to originate in the cerebellum and involve the cerebellum-thalamic-cortical loop (Lenka et al., 2017; Raethjen and Deuschl, 2012). The DCN and IO are the outputs and inputs of the cerebellum, respectively, and they are interconnected. In particular, synchronous activity in the IO is thought to be a generator of ET (Handforth, 2012). Expression of netrin-G2 is distributed in various regions of the telencephalon and multiple areas of cerebellar circuits, such as the DCN in the olivocerebellary circuitry, cerebellar cortical Bergmann glia, and IO (Fig. 1C) (Nishimura-Akiyoshi et al., 2007; Yaguchi et al., 2014). However, netrin-G2 is widely expressed in several brain areas, and the neural circuits involved in the microtremors could not be identified by their distributions.

Harmaline, a monoamine oxidase inhibitor which inhibits the degradation of serotonin and noradrenaline, is thought to generate tremors with effects on the gap junctions in the IO and $\gamma$-aminobutyric acid (GABA)-mediated DCN-IO regulation (Handforth, 2012). Harmaline’s effect on the IO-DCN loop alone is sufficient to generate tremors (Llinas and Volkind, 1973). Furthermore, some anti-tremor drugs for human ET weaken harmaline-induced tremors (Park et al., 2010; Paterson et al., 2009). These are the reasons why harmaline-induced tremor is a model for ET. These findings suggest that possible circuits involved in microtremors of netrin-G2 KO mice also exist in the cerebello-thalamic-cortical loop, similar to that in human ET. Netrin-G2 gene abnormalities in humans, however, result in motor retardation without symptoms of ET (Dias et al., 2019). Further investigation is required to understand the neural circuits underlying the microtremors in netrin-G2 KO mice.

4.3. Comparison between human ET or harmaline-induced tremors and microtremors in netrin-G2 KO mice

Alcohol can relieve ET symptoms in patients (Growdon et al., 1975; Mostile and Jankovic, 2010) and the frequency of the tremors remains the same (Koller and Biary, 1984). The microtremor intensity in netrin-G2 KO mice was not ameliorated by ethanol (Fig. 6A); however, the tremor frequency was shifted to a lower value (Fig. 6B). Tremors in human patients are exacerbated by psychological or physical stress (Handforth and Parker, 2018). In mice, high stress can be induced by forced swim (Commons et al., 2017). Our forced-swim experiment resulted in a significant enhancement of tremors in the KO mice (Fig. 6C), whereas no tremors developed in WT mice. It is more likely that the intrinsic tremors were enhanced by the stress rather than by the shivering induced by lower body temperatures, because the body temperatures of both mice groups changed in a similar way (Fig. S3). Our results suggest that tremors in netrin-G2 KO mice are enhanced by the mice’s vulnerability to stress.

Propranolol, a $\beta$-adrenergic blocker, suppresses harmaline-induced tremors by inhibiting the locus cereuleus-noradrenergic pathway (Paterson et al., 2009). Zonisamide, a T-type calcium channel blocker, and MK-801, a blocker of the NMDA receptors, also suppresses harmaline-induced tremors by inhibiting the pacemaker function in the IO (Park et al., 2010; Paterson et al., 2009). In this study, propranolol and zonisamide treatment did not suppress microtremors in netrin-G2 KO mice, but MK-801 did (Fig. 7A and D). Furthermore, only zonisamide decreased the frequency value of the microtremors (Fig. 7B and E). These results suggest that there are mechanistic differences between microtremors in netrin-G2 KO mice and human ET or harmaline-induced tremors.

5. Conclusions

In this study, we quantified the microtremors in netrin-G2 KO mice using our newly developed tremor detection system. We also examined the effects of alcohol, stress, and pharmacological treatments, known to be influencers of human ET, on the microtremors. We could not demonstrate that these have exactly the same effects on the microtremors in netrin-G2 KO mice as they would on human ET. The classification of human ET has heterogeneities, including various types of tremors which are based on both symptoms and underlying mechanisms. Further studies are needed to clarify the classification of tremors using model animals such as netrin-G2 KO mice. For a quantitative analysis of the tremors of model animals, our tremor detection system will be quite useful. These investigations could lead to the development of therapies for each tremor symptom.
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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jneumeth.2021.109074.

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Declaration of Competing Interest

The tremor detection system was developed by T.Y. and the detection algorithm was patented in Japan (JP 6,495,731). The authors are willing to provide the system to any colleague interested, under a proper MTA.

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