

Brief Reports

Development of Harmaline-induced Tremor in a Swine Model

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Abstract

Background: In the field of translational neuroscience research, it is critical to utilize a large animal model to test the feasibility, safety, and functionality of novel therapies. Here, we describe a protocol for the development of a large animal model of tremor.

Methods: In a pig model, tremor was induced with harmaline and measured with wireless accelerometers attached to the limbs. Three different doses of harmaline were tested and three repetitive injections were made at 72-hour intervals. To fully characterize the drug-induced tremor, onset time, tremor amplitude, maintained duration, and peak tremor frequency were analyzed.

Results: Harmaline-induced tremor appeared immediately following intravenous injection of harmaline. Tremor was maintained over 2 hours. Its frequency was 10–16 Hz, which was independent of doses. Dose-dependent responses were observed in tremor amplitude, triggering time, and tremor-maintained duration. Repetitive injection of harmaline desensitized the harmaline effect.

Discussion: We provide a detailed protocol for training, drug injection, device selection, and tremor recording optimized to create a swine model of tremor with harmaline. Our protocol provides reliable tremor in pigs and suggests pig as a valid translational large animal model of tremor.

Keywords: Harmaline, tremor, pig, animal model, wireless accelerometer

Citation: Lee J, Kim I, Lee J, Knight E, Cheng L, il Kang S, et al. Development of harmaline-induced tremor in a swine model. *Tremor Other Hyperkinet Mov.* 2018; 8. doi: 10.7916/D8J68TV7

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Editor: Elan D. Louis, Yale University, USA

Received: November 30, 2017 **Accepted:** February 5, 2018 **Published:** March 13, 2018

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Funding: This study was funded by the National Institutes of Health, National Institute of Neurological Disorders and Stroke (NS 88260).

Financial Disclosures: None.

Conflicts of Interest: The authors report no conflict of interest.

Ethics Statement: All performed experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Introduction

Pigs have been broadly and increasingly used as a large animal model in the biomedical and translational research fields due to the similarity of their internal organ anatomy and biochemical physiology to humans. Recently, pigs have also increasingly been used in the neuroscience and biobehavioral research fields because pigs have a compatible size and volume of the brain to non-human primates; and the anatomy and neurodevelopment of the pig brain is similar to the human brain. The swine brain thus can accommodate multimodality imaging and the surgical installation of multiple electrodes, similar to that used for humans.¹ Multiple neurologic models have been

developed and tested including Parkinson's disease,² traumatic brain injury,³ and stroke.⁴ However, the essential tremor (ET) model has not yet been fully examined in pigs or other large animal models.

ET is the most frequent form of pathologic tremor and one of the most common adult-onset neurologic impairments.⁵ The incidence rate for people age 60 and older is estimated at 6.3–9%.⁶ It affects approximately 10 million people in the United States.⁵ Despite its high prevalence and disabling effects, pharmacological intervention only helps about 50% of patients with ET; and nearly one out of three patients stops taking their medications.⁵ The development of new therapies has been hampered by a lack of knowledge about tremor pathophysiology and the lack of a validated preclinical model for

evaluating potential tremor-suppressing drugs. Therefore, it is important and necessary to develop an animal model of ET to investigate underlying mechanisms and pathophysiology of ET and screen novel drugs and other therapeutic options.

Harmaline, a β -carboline derivative, is widely used for experimental analysis of tremor; and a rodent model of harmaline-induced tremor has been used as a model of ET.⁷ In the rodent tremor model, the tremor frequency and responsiveness to propranolol, ethanol, and octanol are similar to that of humans with ET.⁷⁻⁹ Harmaline induction of a fine, generalized 8–12 Hz tremor has also been demonstrated in sheep and non-human primates.^{10,11} However, harmaline-induced tremor has not been tested in pigs.

One reason why the harmaline-induced tremor model is regarded as the best model of ET is that the olivocerebellar system plays a key role in pathophysiological mechanisms underlying both harmaline-induced tremor in animals^{7,9} and ET in humans.¹² Thus, harmaline-induced tremor in animal models may share underlying mechanisms relevant to the understanding of tremor generation in human ET.¹² For this reason, a rodent harmaline-induced tremor model was developed and used to investigate tremor pathophysiology. However, considering the substantial anatomical differences between the rodent and human brain, rodent models have limitations.⁹ The pig brain is gyrencephalic and more closely resembles the human brain in anatomy and development compared to the rodent brain.¹ Therefore, here, we describe a protocol for development and characterization of a harmaline-induced tremor in the large animal pig model.

Methods

Animals

Yucatan mini-pigs (two) and domestic pigs (five) were used. Animals were socially housed in pairs and maintained in accordance with the Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), international-accredited facility. Swine cages were cleaned daily and sanitized weekly before feeding. The animals were fed standard chow (Purina Animal Nutrition, LLC, Shoreview, MN) as recommended by the attending veterinarian and provided ad libitum access to tap water. For completeness, enrichment was provided in the form of twice weekly rotation of toys and daily treats. Records of cleaning schedules as well as temperature and humidity recordings were monitored daily. Each animal was checked daily by the animal care staff and 3 days per week by the Mayo Clinic veterinarians. All experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Acclimation to tremor-monitoring procedures

Pigs are intelligent animals that sensitively respond to external stimuli and are relatively easy to train. Before the harmaline injection, animals were acclimated to the tremor-monitoring room, experimental condition, the accelerometer attachment on the limb, and experimenter (Figure 1A). A small empty space (~10 ft × ~15 ft) was dedicated for tremor monitoring using fences to limit the space. For the acclimation

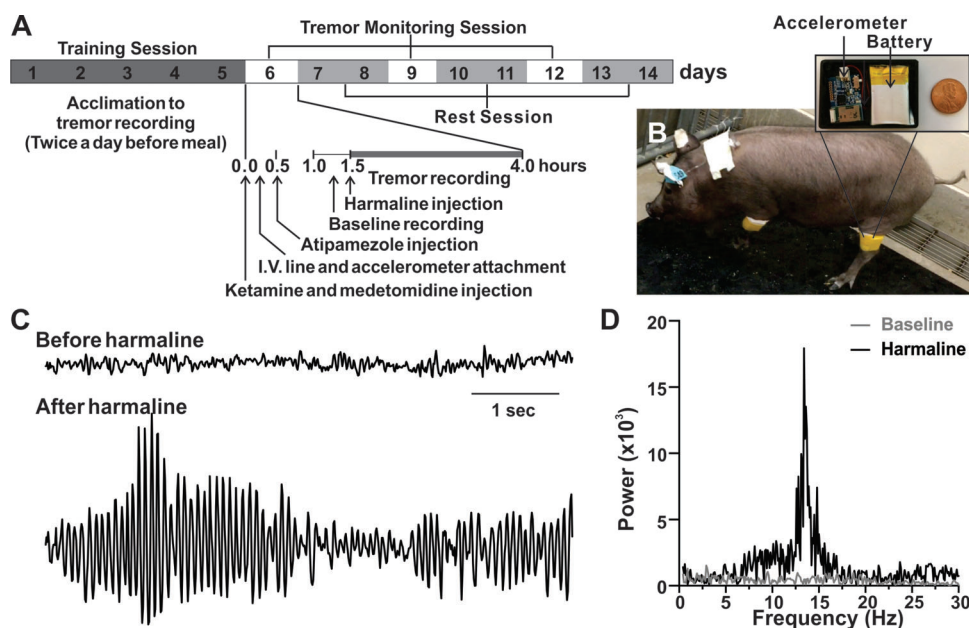


Figure 1. Harmaline-induced Tremor Model in Pig. (A) Diagram of experimental design. Animals were acclimated to the accelerometer attachment and experimental environment before the recording session. Harmaline was repetitively injected three times at 2-day intervals. During the recording session, baseline (for 30 min) was recorded first and harmaline was injected through the intravenous (IV) line in the ear. (B) Photo of device attachment. Accelerometers and the IV line were attached and maintained throughout the recording session. (C) Raw tremor traces. Tremors were detected through the accelerometer attached on the limb. Rhythmic motion was significantly increased by harmaline injection (6 mg/kg). (D) Harmaline-induced tremor frequency. The predominant tremor frequency induced by harmaline is 10–16 Hz in pig. There is another significant tremor detected at 8–10 Hz.

to the recording environment and tremor-monitoring procedure, animals were guided to voluntarily walk to the monitoring room, and two empty containers of accelerometers with a weight matched to the actual device (15.6 g in Figure 1B, inset) were attached to the limbs (Figure 1B). The accelerometer was attached while the animal was distracted by a treat and water. The accelerometer was placed onto the limb and then wrapped with an elastic vetband (VetOne, Escondido, CA) around the limb. Once the accelerometer was attached to the limb, animals were allowed to freely move around for 15–30 minutes. This acclimation procedure was performed prior to feeding twice a day, up to 5–7 days. While the pig was in the monitoring room, the pig was provided treats, water, and regular chow. After the acclimation procedure, the animal was walked back to its home cage. Once it completed the procedure, it received a fruit snack and regular chow as a reward.

Harmaline-induced tremor

Once the animal was acclimated to the accelerometer, harmaline-induced tremor monitoring was performed. On the day of the experiment, the pig was temporarily sedated with a combination of ketamine (10 mg/kg) and medetomidine (0.2 mg/kg) intramuscularly. A venous indwelling catheter was inserted into an ear vein and the accelerometer was attached to its limbs (Figure 1B). Before the accelerometer was attached, the skin was cleaned with water and hairs were shaved with a clipper. Then, the skin was covered with ioban drapes (MMM, St. Paul, MN). The accelerometer was attached on top of the ioban drape-covered area using a dressing bandage and was wrapped with an elastic vetband (Figure 1B). Once all equipment was attached, the pig was awakened by atipamezole administration (1 mg/kg intravenously). The animal would typically regain consciousness and stand within 5–10 minutes. However, we waited for approximately 60 minutes to allow the animal to fully recover from the anesthesia and ambulate normally. Once the animal could walk normally and maintain balance (as determined by the ability to respond quickly to a mild push), a baseline walk was monitored with the attached accelerometer for 30 minutes. Then, harmaline was administered intravenously at a dose of 2.5, 5, and 6 mg/kg dissolved in sterile saline (10 mL). Harmaline (harmaline hydrochloride, Sigma-Aldrich, St. Louis, MO) is a yellow powder and saline with harmaline is yellow. Owing to its color, we could easily visualize the harmaline solution in the intravenous (IV) line and determine the injection time. We freshly prepared harmaline solution immediately before the injection. Because the fully awake animal was responding to the saline injection through the venous indwelling catheter in the ear, extra caution was required. Once the prepared harmaline saline was injected, the IV line was cleared with saline (saline flush) to deliver the full dose of harmaline to the animal. Accelerometer monitoring was performed for a maximum of 3 hours. Harmaline-induced tremor monitoring was repeated up to three times at 72-hour intervals. A timeline of the experiment is shown in Figure 1A.

Wireless movement sensor

To perform consistent and reliable measurement of tremor over time, we built a band-type inertial measurement unit (IMU), which consisted of an MSP430 microcontroller (MSP430F1611, Texas Instruments, San Diego, CA) and low-cost inertial sensors of a three-axis accelerometer and magnetometer (LSM303DLM, STMicroelectronics, Geneva, Switzerland). A rechargeable battery (PSE H503438-PCM [3.7 V, 680 mAh], PSE, Jinan, China) was embedded and guaranteed approximately 8 hours of operation. The IMU was designed to sense ± 8 g acceleration and the sampling rate was set at 100 samples/second. Sensing-data parameters were preprocessed in the microcontroller and transmitted via the embedded Bluetooth module (Parani-ESD200, Sena Technologies Inc., Seoul, Korea) to a portable computer. The data acquisition was controlled by a custom-made PC program. For tremor detection and analysis, three-axis accelerations (x [n], y [n], and z [n], where n is the sample) were used.

Tremor measurement

Motion activity was measured with an in-house fabricated wireless movement sensor (Figure 1B, inset). Motion power was transmitted wirelessly and recorded digitally. The device was calibrated by dropping a 2.75-g ping-pong ball 5.7 cm and adjusting the amplifier sensitivity so the recorded signal was 1 cm on an oscilloscope set at 1 V/cm. Data were sampled at 100 Hz, collected in 5-second bins, and exported to MatLab 2016b (MathWorks, Natick, MA) (Figure 1C). In MatLab, fast Fourier transform (FFT) was performed on amplitude of accelerometer data in the time domain to obtain power spectra in the frequency domain (Figure 1D). The total motion power for each 10-minute epoch was calculated between 0 and 10 Hz (non-tremor motion spectrum) and between 10 and 16 Hz (the harmaline tremor frequency bandwidth), and the motion power ratio (MPR) then was calculated.

$$\text{MPR} = (10\text{--}16 \text{ Hz power}) / (0\text{--}10 \text{ Hz power})$$

Each 10-minute epoch was divided into 5-second bins to calculate MPR and each of these MPR calculations were normalized using the averaged MPR of the 30 minute baseline (Figure 2A,B).

Tremor analysis

To fully characterize harmaline-induced tremor, tremor was analyzed into four categories: 1) latency of tremor onset; 2) tremor amplitude; 3) maintained duration, which is the duration until the tremor is gone; and 4) peak tremor frequency (Figure 2C). The data were divided into 1-second time frames and FFT performed to determine the MPR. To find significant tremor frames, Gaussian fit was performed to the MPR of each frame and frames that had a p-value less than 0.01 were selected. We define the onset time as the time from the end of the IV push and flush to the time at which tremor criteria was met as described above. Tremor amplitude was measured as root-mean-square of 5-minute window where the maximum values were obtained between 60 and 90 minutes after harmaline injection. Maintained duration was defined by the time between the first frame and the last frame of

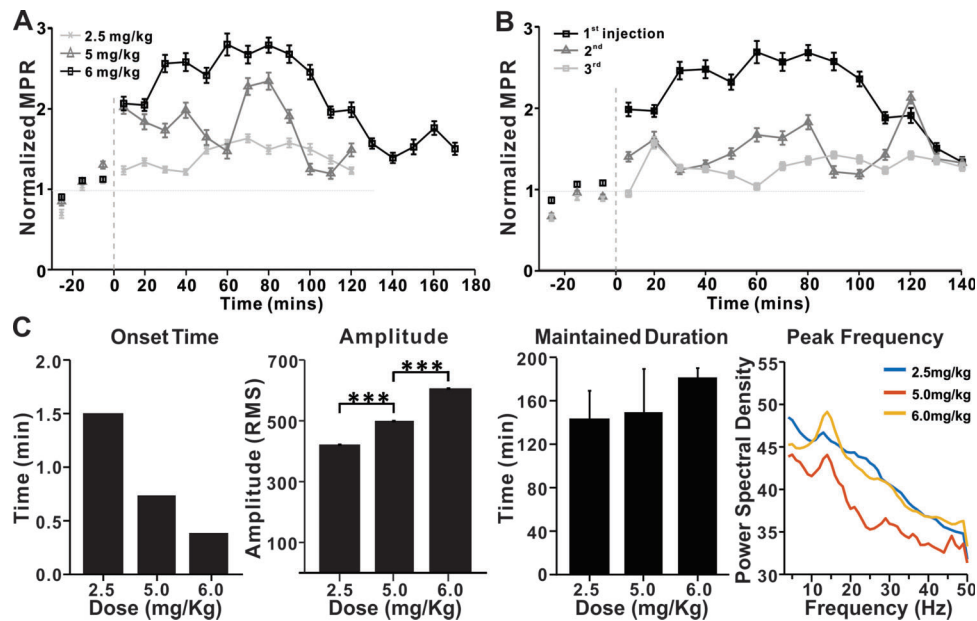


Figure 2. Dose Dependence and Desensitization to Harmaline Effect. (A) Dose dependency of harmaline effect. Motion power ratio (MPR) was calculated by tremor with 10-minute epochs. The normalized MPR was obtained by dividing each MPR by the average of baseline MPR. Harmaline-induced tremor was dependent on the concentration of harmaline. Three different concentrations of harmaline were tested in pigs. The low concentration of harmaline (2.5 mg/kg) showed weaker MPR than the two higher concentrations (5.0 and 6.0 mg/kg) of harmaline. Light-grey dashed line indicates the point of harmaline injection. The error bars represent the standard error of the mean. (B) Desensitization to harmaline effect. The harmaline effect (6 mg/kg) was reduced by repetitive injections of harmaline. At the third injection, the onset-time of tremor induction was significantly delayed (70 minutes) and the maintained duration was decreased by 73.9 min (maintained only for 98.3 minutes). (C) Dose dependency of harmaline-induced tremor. Onset time of tremor, amplitude, maintained duration and frequency profile were analyzed. * $p < 0.05$, *** $p < 0.0001$.

significant tremor detection. Using MPR, we characterized the temporal profile of harmaline-induced tremor. A total of seven pigs were used to optimize the procedures. Three concentrations of harmaline (2.5, 5.0, and 6.0 mg/kg) were used to characterize the harmaline-induced tremor profile ($n = 2, 2$ and 3 , respectively). Since harmaline effect is attenuated by repetitive injections, consistent with previous rodent data,¹³ harmaline (6.0 mg/kg) was injected three times, 72 hours apart ($n = 3$).

Results

Harmaline induced significant motion tremor in the pigs (Figure 1C). Tremor was apparent both during laying and standing;¹⁴ however, it was enhanced during movements such as standing and walking (Figure 3).¹⁵ The amplitude of raw data before and after harmaline injection is shown in Figure 1C. The tremor frequency was 10–16 Hz (Figure 1D). The time course of mean MPR of the harmaline-induced tremor is shown in Figure 2A,B. The overall averaged MPR of the first harmaline injection was dose dependent (mean MPR, 1.34, 1.57 and 1.93; 2.5, 5, and 6 mg/kg of harmaline, respectively; $p < 0.001$ by analysis of variance [ANOVA]) (Figure 2A). The harmaline-induced tremor was robust; however, its amplitude and appearance was irregular and both decreased and slowed in a nonlinear manner throughout the whole experiment period. To characterize harmaline-induced tremor, four parameters were analyzed: onset time, amplitude, total tremor period, and the peak tremor frequency.

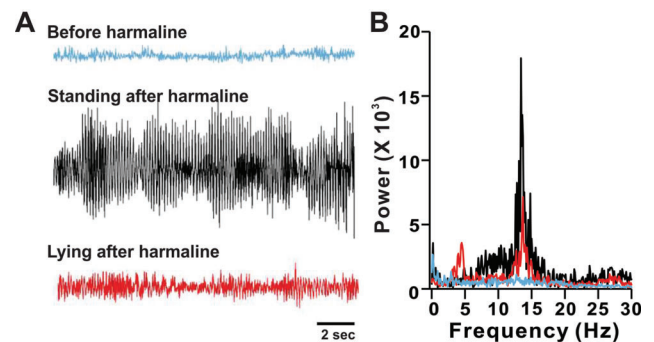


Figure 3. Comparison of Harmaline-induced Tremor Between Standing and Lying. (A) Raw tremor traces. Rhythmic motion induced by harmaline (6 mg/kg) was significantly larger while standing compared to lying down. (B) Harmaline-induced tremor frequency. Power spectrum analysis was performed.

Harmaline initiated tremor in a concentration-dependent manner. High concentration (6.0 mg/kg) harmaline induced the tremor almost immediately after harmaline injection (0.4 min), whereas low concentration harmaline (2.5 mg/kg) had a delayed tremor onset time (1.5 min) (Figure 2C, onset time of tremor). We also compared the total duration of harmaline-induced tremor at the first round of harmaline injection: tremor with 2.5 mg/kg was maintained for 125.2 ± 18.2 min; with 5 mg/kg, 120.93 ± 28.2 min; and with 6 mg/kg, 172.6 ± 5.1 min (Figure 2C, maintained duration). We separated and analyzed the

amplitude of tremor: 1.08 ± 0.03 , 1.25 ± 0.04 , and 1.34 ± 0.04 seconds (mean \pm standard error of the mean; 2.5, 5, and 6 mg/kg, respectively), which is dose-dependent (Figure 2C, tremor amplitude; 2.5 mg/kg vs. 5 mg/kg, $p < 0.0001$; 2.5 mg/kg vs. 6 mg/kg, $p < 0.0001$; 5 mg/kg vs. 6 mg/kg, $p < 0.0001$ by t-test). The peak tremor frequency was also analyzed and it was consistent throughout three different concentrations of harmaline (Figure 2C, Peak tremor frequency; 13, 14, and 14 Hz with 2.5, 5, and 6 mg/kg).

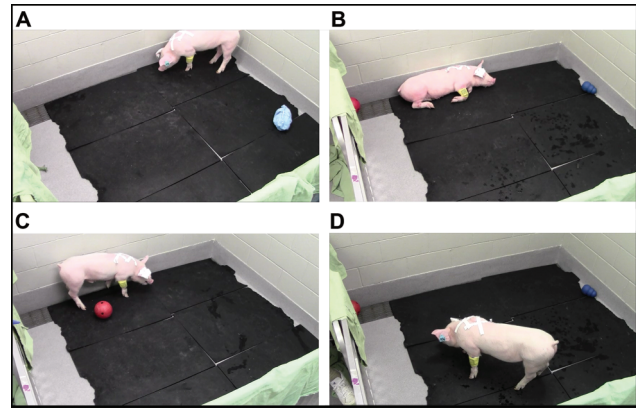
In the previous study with rodents,¹³ the repeated administration of harmaline showed a progressive loss of drug-induced tremor. Therefore, we also examined the pattern of harmaline tolerance by repetitive injection. To minimize tolerance, we performed harmaline injection at 48-hour intervals. There was a significant decline in mean MPR with repeated injections. The mean MPR of total duration after harmaline (6 mg/kg) administration from the first injection throughout the third injection was 1.87, 1.38, and 1.18, respectively (Figure 2B; $p < 0.001$ by ANOVA).

Discussion

Here we demonstrated an experimental method to develop a harmaline-induced tremor model in the pig. We used wireless accelerometers, which can also be used for humans (Figure 1B), and observed postural and action tremors. MPR was calculated and used for analysis and three doses of harmaline were tested. To fully characterize the harmaline-induced tremor, onset time, amplitude, total tremor period, and the peak frequency were identified, and these parameters except frequency were affected in a concentration-dependent manner (Figure 2A,C). In previous studies, repeated administration of harmaline in rats led to the development of desensitization of tremorogenic effects of harmaline.¹³ In pigs, repetitive harmaline injection reduced the severity of harmaline-induced tremor, which coincides with previous studies (Figure 2B).^{7,8}

Advantages of the large animal tremor model

ET and other tremor disorders have a tremendous impact on patients' quality of life, and clearly warrant further study into the mechanisms by which they arise and options for effective tremor suppressing therapy. As noted in the introduction, both basic and preclinical studies have previously employed a small animal model of harmaline-induced tremor. While these models have provided some insight into the pathophysiology and treatment of tremor,⁸ rodent models are limited by the substantial anatomical differences between rodent and human thalamic functions (e.g., the rodent thalamus lacks interneurons).^{9,16} However, to advance further along the translational research spectrum it is critical to have a model, such as the pig, that more closely resembles the human brain in anatomy and development compared to the rodent brain.¹ Furthermore, given that the swine brain is comparable in size and volume to the non-human primate brain, it is large enough to accommodate multimodality imaging and the surgical insertion of multiple electrodes. As a result, the swine model can be used to investigate the mechanisms by which tremor



Video 1. Harmaline-induced Tremor (6 mg/kg, i.v. injection). (A) Before injection. (B) Lying after harmaline injection. (C) Walking 30 minutes after harmaline injection. (D) Walking around 90 minutes after harmaline injection.

arises and to screen drugs and other potential therapeutic interventions for efficacy.

Behavioral manifestations of harmaline-induced tremor

Since a large animal has been used in this study, several other behavioral and physiological changes could be easily detected. Tremor mostly started whole body and limbs and maintained at the limbs for a long time (Video 1). For the first 30–60 min of harmaline infusion, some animals displayed whole body trembling, agitation, barking and falling, which were dependent upon the harmaline dosage. At this stage (Video 1), the skin often turned to red due to hyperthermia. One animal (out of seven) showed emesis with 6 mg/kg of harmaline at the first injection; however, other pigs did not show this type of reaction. Pigs are not in-bred species, and thus may demonstrate greater individual variability.

Limitations of future directions

While this is a promising model for future studies, we acknowledge some limitations of the present study. First, harmaline was infused intravenously. Thus, it can affect central and peripheral nervous systems as well as other systems in the body, which may cause direct or indirect autonomic physiological changes. To isolate the central nervous system-involved tremorgenesis, microinjection targeting a specific brain area needs to be developed and tested. In addition, since the infusion speed and circulation cannot be controlled experimentally with this IV infusion method, these may confound the precision on our measurements of onset time.

Second, in ET and other action tremors in humans, tremor is measured while the posture of the human with tremor is well controlled (e.g., sitting on a chair, or walking slowly). We observed that tremor was more noticeable during standing and walking compared to lying down (Figure 3 and Video 1). In the future, we also need to consider standardization of the animal posture and activities, which will allow for more specific quantification of action vs. rest components of a harmaline-induced tremor.

We observed a similar desensitization phenomenon to that described in the rat model. In rats treated with harmaline, this effect has been associated with extensive loss of Purkinje cells in parasagittal bands of cerebellar cortex.^{9,17} Because this will limit the number of times an individual animal could be used for drug or device testing, use of the pig model is acknowledged to be resource intensive. However, it is also possible that more extended rest periods than those employed in this study may delay desensitization further.

We also noted an interesting bimodal response in the harmaline-induced tremor amplitude in both the 5 mg/kg dose condition and in the second injection of 6 mg/kg of harmaline. We could not generate a definitive physiologic explanation for why this bimodal response may occur. To determine the reliability of this finding and test hypotheses for why it may occur, a larger sample size would be needed.

Finally, here we have tested and characterized the harmaline-induced tremor in pigs. While it is important to note that the harmaline-induced tremor model in the pig does not directly model the underlying disease pathophysiology of ET, it potentially involves some of the same underlying neural circuits.^{7,8,12} However, due to its faithful reproduction of action tremor in a large animal model, this model will be valuable in future research to better understand the underlying neurophysiology contributing to ET and other action tremors, as well as the mechanism by which different therapeutic interventions ameliorate tremor. The harmaline-induced swine tremor model developed here could become a platform for preclinical testing of new drugs and novel devices for tremor and translational research and also could become a more general tool for basic research.

Conclusion

We provide an experimental behavior protocol to develop a drug-induced tremor model in a large animal, pig, and suggest this as a valuable preclinical model to better understand the underlying neurophysiology contributing to ET and other action tremors, as well as the mechanism by which different therapeutic interventions ameliorate tremor.

Acknowledgement

We sincerely thank Drs. Jodi A. Scholz and Michael C. Blanco, Mayo Veterinarians, for their clinical and practical advice on large animal behavior protocol and support. This study was funded by the National Institutes of Health, National Institute of Neurological Disorders and Stroke (NS 88260).

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