Video Article

# Gait Analysis of Age-dependent Motor Impairments in Mice with Neurodegeneration

Christine M. Rostosky[1,2], Ira Milosevic[1,2]

[1]European Neuroscience Institute (ENI)

[2]University Medical Center Göttigen (UMG)

Correspondence to: Ira Milosevic at imilose@gwdg.de

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## Abstract

Motor behavior tests are commonly used to determine the functional relevance of a rodent model and to test newly developed treatments in these animals. Specifically, gait analysis allows recapturing disease relevant phenotypes that are observed in human patients, especially in neurodegenerative diseases that affect motor abilities such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and others. In early studies along this line, the measurement of gait parameters was laborious and depended on factors that were hard to control (*e.g.,* running speed, continuous running). The development of ventral plane imaging (VPI) systems made it feasible to perform gait analysis at a large scale, making this method a useful tool for the assessment of motor behavior in rodents. Here, we present an in-depth protocol of how to use kinematic gait analysis to examine the age-dependent progression of motor deficits in mouse models of neurodegeneration; mouse lines with decreased levels of endophilin, in which neurodegenerative damage progressively increases with age, are used as an example.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57752/

## Introduction

Neurodegenerative diseases impose a significant burden on patients, families, and society, and will become of even greater concern as life expectancy increases, and the world population continues to age. One of the most common symptoms of neurodegenerative diseases are balance and mobility problems. Thus, characterization of motor behavior in aging mammalian (*e.g.,* rodent) models, and/or models showing neurodegenerative phenotypes, is a valuable tool to demonstrate the *in vivo* relevance of the specific animal model(s), or therapeutic treatments that aim to improve the disease symptoms. Almost every approach to treat neurodegenerative diseases ultimately requires testing in an animal model before initiation of a clinical trial in humans. Therefore, it is crucial to have reliable, reproducible behavior tests that can be used to consistently quantify disease-relevant phenotypes along age progression, in order to ensure that a candidate drug, which showed potential in an *in vitro* model, can effectively ameliorate the phenotype in a living animal.

One aspect of motor behavior assessment in rodents is kinematic gait analysis, which can be performed by VPI (also called ventral plane videography)[1,2]. This established method capitalizes on continuous recording of the underside of the rodents walking atop a transparent and motorized treadmill belt[1,2,3,4]. Analysis of the video feed data creates "digital paw prints" of all four limbs that dynamically and reliably recapitulate the rodent's walking pattern, as originally described by Kale *et al.*[2] and Amende *et al.*[3].

The principle of imaging-based gait analysis is to measure the paw area in contact with the treadmill belt over time, for each individual paw. Every stance is represented by an increase in paw area (in the braking phase) and a decrease in paw area (in the propulsion phase). This is followed by the swing phase in which no signal is detected. Swing and stance together form a stride. In addition to gait dynamics parameters, posture parameters can also be extracted from the recorded videos. Exemplary parameters and their definition are listed in **Table 1** and include stance width (SW; the combined distance from the fore or hind paws to the snout-tail axis), stride length (SL; average distance between two strides of the same paw), or paw placement angle (the angle of the paw to the snout-tail axis). The posture and gait dynamics data allow drawing conclusions on animal balance (by posture parameters and their variability over several steps) and coordination (by gait dynamics parameters). Other parameters, such as ataxia coefficient (the SL variability calculated by [(max. SL−min. SL)/mean SL]), hind limb shared stance time (time that both hind limbs are in contact with the belt), or paw drag (total area of the paw on the belt from full stance to paw lift-off) can also be extracted, and have been reported to be changed in various neurodegenerative disease models[5,6,7,8] (see **Table 1**).

| Parameter | Unit | Definition |
|---|---|---|
| swing time | ms | duration of time the paw is not in contact with the belt |
| stance time | ms | duration of time the paw is in contact with the belt |
| % brake | % of stance time | percentage of stance time the paws are in the brake phase |
| % propel | % of stance time | percentage of stance time the paws are in the propulsion phase |
| stance width | cm | combined distance from the fore or hind paws to the snout-tail axis |
| stride length | cm | average distance between two strides of the same paw |
| stride frequency | strides/s | number of complete strides per second |
| paw placement angle | deg | angle of the paw in relation to the snout-tail axis of the animal |
| ataxia coefficient | a.u. | SL variability calculated by [(max SL-min SL)/ mean SL] |
| % shared stance | % of stance | hind limb shared stance time; time that both hind limbs are in contact with the belt at the same time |
| paw drag | $mm^2$ | total area of the paw on the belt from full stance to paw lift-off |
| limb loading | $cm^2$ | MAX dA/dT; maximal rate of change of paw area in the breaking phase |
| step angle variability | deg | standard deviation of the angle between the hind paws as a function of SL and SW |

**Table 1. Definition of key gait parameters that can be tested by ventral plane imaging.**

Assessing the motor behavior of rodent models for neurodegenerative diseases can be challenging depending on the severity of the phenotype of a specific model at a given age. Several diseases, most prominently PD, show strong motor behavior (locomotion) deficits, both in patients and in animal models. One of the four key symptoms in PD is bradykinesia, which progresses with aging and manifests in severe gait impairments already in early stages of PD[9]. Studies of the acute PD model, rodents treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP), have already used VPI gait analysis[10,11,12]. However, given the acute nature of this model, these studies do not address the age-related progression of motor deficits. Several recent studies have conducted gait analysis in aged mice with neurodegenerative changes, for example[13,14,15], emphasizing the relevance of understanding the disease progression with advancing age.

In addition to motor deficits, animal models of neurodegenerative diseases often have difficulties focusing on the examination tasks and show prominent cognitive impairments, in particular with advancing age. Such a phenotype can influence the result of motor behavior tests. Namely, one of the most widely used tests to examine motor deficits, the rotarod test[16], relies on cognition, attention, and stress[17,18]. While the willingness to walk on a motorized treadmill also depends on these factors, the recorded read-out is running, which is a more standardized feature and far less influenced by altered cognition. Effects of stress and attention may be visible in specific parameters, like swing/stance time for stress, and SL for attention[19,20], but not in overall running ability.

The kinematic gait analysis approach further offers the advantage of having options to adjust the challenge for rodent models. The treadmill with adjustable angle and speed allows walking speeds from 0.1 - 99.9 cm/s, so that rodents with severe walking impairments may still be able to run at a slow speed (~10 cm/s). Non-impaired animals can be measured at faster running speeds (30 - 40 cm/s). The observation of whether or not the tested animals are able to run at a certain speed provides a result by itself. Further, the rodent can be additionally challenged to run up an incline, or down a decline, by tilting the treadmill to a desired angle with the help of a goniometer, or by attaching a weighted sled to mouse or rat hind limbs.

In addition to numerous studies of single proteins that are mutated in patients, there is a recent increasing awareness of the links between defective endocytosis process and neurodegeneration[13,21,22,23,24,25,26,27,28]. Mouse models with reduced levels of endophilin-A (henceforth endophilin), a key player in both clathrin-mediated endocytosis[13,21,29,30,31,32,33,45] and clathrin-independent endocytosis[34], were found to show neurodegeneration and age-dependent impairments in locomotor activity[13,21]. Three genes encode the family of endophilin proteins: endophilin 1, endophilin 2, and endophilin 3. Notably, the phenotype resulting from depletion of endophilin proteins varies greatly depending on the number of missing endophilin genes[13,21]. While triple knock-out (KO) of all endophilin genes is lethal just a few hours after birth, and mice without both endophilin 1 and 2 fail to thrive and die within 3 weeks after birth, single KO for any of the three endophilins shows no obvious phenotype for tested conditions[21]. Other endophilin mutant genotypes show reduced lifespan and develop motor impairments with increasing age[13]. For example, endophilin 1KO-2HT-3KO mice display walking alterations and motor coordination problems (as tested by kinematic gait analysis and rotarod) already at 3 months of age, while their littermates, endophilin 1KO-2WT-3KO animals, display a significant reduction in motor coordination only at 15 months of age[13]. Due to the vast diversity of phenotypes in these models, it is necessary to identify and apply a test

that can integrate a variety of challenges corresponding to the animal's motor and cognition abilities, as well as the age. Here, we detail the experimental procedures that capitalize on the kinematic gait analysis to assess the onset and progression of motor impairments in a mouse model that shows neurodegenerative changes (*i.e.,* endophilin mutants). This includes measuring gait parameters at various ages and different severities of locomotion impairments.

## Protocol

All animal experiments reported here are conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), registration number 14/1701.

# 1. Study Design

1. As animal behavior work requires careful planning, consider the following parameters while designing the experiment.
    1. Number of animals needed per group.
        1. Use a statistical software (*e.g.,* PASS, EDA, or GPower) to calculate the required group size.
        NOTE: The group size depends on the variation between animals and the severity of the phenotype. For the kinematic gait analysis, the number of mice is usually 10 - 20 per group.
    2. Sex of the experimental animals.
        1. Consider the effect of estrogen levels on the experiment, depending on the animal strain.
        NOTE: Many behavior studies focus on males in order to avoid the influence of estrogen levels on the experiment. These influences are more or less strong depending on the animal background strain.
        2. If both sexes will be used, test for sex influence and evaluate the two sexes independently when necessary.
    3. Age of the experimental animals.
        1. Use adult animals (2 months of age, or older) if only one-time point is needed.
        2. Select several time points when change in motor behavior with advancing age is to be studied. The earliest possible time point is 1 month, after mice are weaned from their mothers. Test the animals in regular intervals, *e.g.,* every 1, 2, or 3 months.
2. Apply for authorization from the local authorities to perform animal behavior testing.
3. Make plans for procuring the test animals.
    1. Make a breeding plan or contact an animal distributor in a timely manner so that enough experimental animals are available on the day when the experiments start.
    2. Allow the animals to habituate for one week if they are kept in a new room/setting during the experiments.

# 2. Video Recording

NOTE: To illustrate the use of kinematic gait analysis, here a commercially available imaging system with its accompanying imaging and analysis software (see the **Table of Materials**) are used.

1. Start the computer and the imager software.
2. Determine the health status and well-being of each animal by observing it in its home cage, and weighing it on a balance.
3. When needed, gently apply red finger paint to the animal's paws with a brush. Allow the paint to dry for ~5 min in a spare clean cage.
NOTE: Avoid painting the animal's abdomen as the paint is used to enhance the contrast between paws and body. It is useful to have black finger paint handy for corrections. This step is needed for animals with brown fur, or in case the paws have been tattooed for identification. If chosen to paint the paws of one animal, all animals in the same group and control group need to be painted as well.
4. Set the speed of the treadmill on the top right panel of the apparatus; If more than one running speed will be applied, start with the slowest speed first.
5. Place the animal in the test chamber (avoid clamping the tail or paws when closing the chamber). Cover the chamber with a dark cloth and allow each animal to adjust for 1 - 2 min.
6. Turn on the light in the test chamber by turning the treadmill light rotary switch to the "on" position. Turn the treadmill rotary switch to "forward" to start the treadmill, then click the "record" button in the imager software.
NOTE: While the treadmill is running, it is important to observe animal performance carefully and constantly: stop the treadmill immediately if the animal cannot keep up with the treadmill speed, or shows secondary symptoms non-related to locomotion (*e.g.,* epileptic seizures). Testing conditions may need to be readjusted.
7. When the animal runs stably (no quick escapes to the sides, front, or back), record for at least 5 s before stopping the treadmill. Stop recording by clicking "stop" on the imager software, and turn the treadmill rotary switch back to the "off" position.
NOTE: To avoid unstable running of animals it can be helpful to let them run for several seconds, or to allow them to run in the other direction (by turning the treadmill rotary switch to "reverse" instead of "forward").
8. Click the "processing" button in the imager software to open a menu in which the start and the end point of the video section (to be used for analysis) can be set. To do this, use the slider on the bottom of the screen to navigate through the video.
9. To select the current time point as the start or end point, click "from frame #" and "to," respectively. Make sure the section contains at least 7 steps/paw (14 steps in total) of the animal running stably at a constant speed.
10. Enter the animal identification, birth date, weight, and sex. Save the data on a desired location on the computer or server. Click "camera" to return to the recording interface.
11. If multiple running speeds need to be recorded, repeat steps 2.6 - 2.10 with the desired running speeds. Before recording the next video, ensure that the red paint is still present on the paw, otherwise repeat step 2.3.

12. After recording, release the animal to its home cage. After removing an animal, clean the treadmill belt thoroughly with soapy water followed by disinfectant to prepare it for the next experimental animal.

## 3. Video Processing

1. Start the analysis software and click "select study folder" to select the folder with the recorded videos.
2. Select one video, or several videos that can be processed consecutively, and click "go."
3. Use the "redraw" function to select the area where the mouse is running; this section should only contain the mouse and white background.
4. If the "reverse" treadmill function was previously used, choose "Check if subject's nose is to your right >>>" to mirror the video since the software is designed to only analyze animals running to the left. Click "accept" to proceed.
5. Use the "refresh" function to see the default mask and paw print that the software detects.
   NOTE: The original video is displayed on the left, and a black and white image of the proposed paw prints is on the right.
6. Enter values in the "length" and "width" boxes to change the mask that excludes the red area around the snout of the animal for analysis; as the color is similar to the paws, not masking that area could result in the software accidentally classifying the snout area as a paw.
7. Adjust the sliders "filter noise" and "filter fur and dark patches" to optimize the black and white paw print. Set the "filter noise" slider to ~800 - 950 for black animals and to ~700 - 800 for brown or white animals, depending on the exact fur color of the animal. Select "ok" when the settings are satisfactory.
   NOTE: The "filter fur and dark patches" slider depends on how "red" the paw is. For painted paws, the value is usually around 100 - 120, and for non-painted paws the best value is around 50 - 100. These settings depend on the color shadings of the fur and paws, and need to be optimized for every animal. The black and white paw print should have clear representations of the paws with as little background noise as possible.
8. Select one or several videos that passed the first adjustment (labeled with "@@" before the video name) and select the "go" function to start the analysis of these videos.
   NOTE: The analysis takes 2 - 5 min per video. It is possible to run the analysis of several videos overnight since this step requires no input from the experimenter.
9. Select an analyzed video (labeled with "@@@") and click "go." Note that the paw area (in $cm^2$) in contact with the belt over time (gait dynamics ) for each separate paw can now be seen. To compare the original video and the calculated paw print for a selected area, use the "play video" function.
10. Use the following (three) tools to correct small mistakes made by the software.
    1. Use the "correct" option to delete a wrong signal, *e.g.,* when the software records a signal even though the corresponding paw is not in contact with the belt. Click once to zoom into the relevant area, and mark the left border of the object to remove with the second click and the right border with the third click.
    2. Use the "connect" option to combine two signals, *e.g.,* when no signal is recorded for a few frames even though the paw is in contact with the belt. Click once to zoom into the relevant area and double-click in the middle of the two objects to combine.
    3. Use the "delete" option to remove time points from the analysis completely. Use this option only if the mistake cannot be fixed with the "correct" or "connect" function, *e.g.,* when a signal from the left forelimb paw is accidentally recorded for the left hind limb paw. Click once to zoom into the relevant area, and mark the left border of the area to remove with the second click and the right border with the third click.
       NOTE: The tools may only be used to correct small mistakes; systematic failures (*e.g.,* if the signal from one paw was extremely weak) cannot be corrected: the video should be excluded from analysis and the recording of the respective animal repeated, when possible. Note that the "play video" option is no longer available after the "correct," "connect," or "delete" option has been used, and clicking the "undo" button will reset all 3 editing tools.
11. Select "next limb" to proceed through the 4 limbs; when "next limb" is clicked after the last paw, the software completes the analysis and shows the results for this animal on 4 screens.

## 4. Gait Analysis

1. When all videos from one experiment are analyzed, select all videos and click "re-organize results" to export the results (a list of parameters in spreadsheet files).
2. Open the file with the ending "reorganized_stride_info" and add information that is not included in this spreadsheet: group information (*e.g.,* genotype, treatment), age, and the measurements of animal length and width which are saved in another spreadsheet file with the ending "SFI_TFI_PFI_reorganized_stride_info."
3. Normalize the gait parameters to animal width or length where necessary, *e.g.,* SL to animal length and SW to animal width.
4. Sort the results by group, age, and running speed: analyze all these conditions independently.
   NOTE: Different ages or running speeds cannot be combined within a same group.
5. Calculate the average (mean) values, standard deviation, and standard error of the mean for each parameter for all experimental conditions.
6. Perform statistical analysis according to the experimental design, *e.g.,* use a 2-tailed *t*-test to compare mutant/treated animal to a wild-type (WT)/control, or ANOVA to compare several independent groups.
7. Look at all measured parameters: it is helpful to plot each parameter to better visualize the results. If there are statistical differences in a given parameter, check if other dependent parameters change correspondingly.
   NOTE: For example, if the SL is significantly decreased in a certain test group, this will also cause a higher stride frequency (since the running speed is the same) and may result in an increased SW (in order to maintain posture stability).
8. Select parameters that are most relevant for a model, and/or are comparable to observations in the human disease. For a presentation, create representative videos for each group and complement them by graphs showing the readout for the relevant parameters, since subtle gait changes are often not obvious from the videos.

## 5. Troubleshooting

NOTE: Some animals, especially mouse models with an anxiety phenotype, may have difficulties to perform even a simple task like running on a treadmill. The following are steps that can be taken to lower anxiety levels and encourage running.

1. Habituation and positive enforcement.
    1. At 2 - 3 days before the first test, place the mouse in the test chamber, cover it with a dark cloth, and leave the light turned off. Let the mouse adjust to the new environment for ~5 min. Add chow or chocolate/nut butter (*e.g.,* Nutella) to the test chamber so a positive association may be formed.

2. Negative enforcement by air puffs/rear boundary.
    1. Mice do not like air puffs or a movement behind them, and will run away from the disturbance. To motivate running, use mild air puffs, or rhythmic movement of the flexible bar that forms the rear boundary of the test chamber, to encourage the mouse to run towards the front part of the test chamber.

3. Slow start.
    1. When testing fast running speeds, start the treadmill at a lower speed and then slowly increase the treadmill speed towards the desired testing condition.

4. Minimize free movement.
    1. The test chamber length is limited by two adjustable bars in the front and back. If a test animal keeps up with the running speed but does not run steadily, limit the chamber's length to result in more steady running.

5. If the above-mentioned measurements are not successful, record running on the next day. If the animal still refuses to run after testing on three days, record this as the finding, and exclude the animal from further testing.
    NOTE: The results of the gait analysis depend on good-quality video recording. There is no reason to exclude videos during the analysis if the videos have been recorded carefully. If the video quality is insufficient, it will become obvious during step 3.6 when the parameters for the creation of the digital paw print are being set. If any other body part except the paws and snout appears red (*e.g.,* due to the missing fur around the genitals or finger paint sprinklings on the abdomen), the quality drops significantly. The adjustments in step 3.6 allow correcting only small issues, and if this cannot bring the video to an acceptable signal/noise ratio, the video needs to be excluded from the analysis, and recording needs to be repeated. Thus, it is recommended to analyze videos soon after recordings are performed.

## Representative Results

To illustrate the use of kinematic gait analysis, we have performed gait analysis on WT C57BL/6J mice with advancing age, as well as several endophilin mutant lines, using commercially available instrumentation and software (please refer to the **Table of Materials**). In this setup, a high-speed camera under a transparent treadmill records the running of a mouse (**Figure 1A**). The software then recognizes the contrast between the red colored paws and the white or black fur. Since our experimental animals had dark brown fur color, we have painted the paws of all subjects with red finger paint. We have tested experimental animals at different running speeds: walking (10 cm/s), running (20 cm/s), and fast running (30 cm/s). The contact area, and time the paws were on the treadmill and in the air were measured. From this information, the parameters that recapitulate gait rhythm (*e.g.,* swing/stance time, brake/propulsion) or posture (*e.g.,* paw angle, SW) were calculated (**Figure 1B**).

We performed gait analysis as a part of a battery of several motor behavior tests. We assessed grip strength (GS), hind limb clasping (HLC), gait, and accelerated rotarod performance (ARR). While motor behavior is not as affected by previous experience and experimental tests as, for example, cognition, it is still important that every animal undergo the same battery of tests in the same order and at the same age. The order should go from low to high difficulty for the animal to minimize influences from previous experiments on the current test.

We have selected endophilin mutants for this study since, depending on how many of the three endophilin alleles are missing, the resulting phenotype varies from no phenotype in the single KOs to a mild neurodegenerative phenotype in young endophilin 1KO-2HT-3KO mice that progresses with aging. For this reason, these animal lines present an adequate model to study subtle changes that develop only as animals age. Given that most endophilin mutants show a reduced lifespan, we have examined the motor behavior of endophilin mutants over the course of 18 months (the 18-month time point was selected since even the mice in the endophilin 1KO-2HT-3KO line that displays the strongest phenotype, do not have paralysis). The gait analysis was performed at eight time points over an 18-month period (**Figure 1C**). At 18 months of age, the animals were euthanized, and preserved for biochemical and/or histological analysis.

**Mouse Colony Maintenance:**

Heterozygous and homozygous mice for the endophilin 1, 2, and 3 alleles were originally reported in Milosevic *et al.*[21] C57BL/6J mice were used in addition to littermate mice as controls throughout. Mice were housed in open cages with *ad libitum* access to food and water in groups of a maximum of 5 animals, on a 12-h light/dark cycle. Only male mice were used in this study to exclude the effects of cycle-dependent variations in females.

**Genotyping of Endophilin A1, A2, and A3 Mouse Models:**

Genotyping of endophilin mutant mice was performed by polymerase chain reaction (PCR) amplification using genomic DNA extracted from tail or ear punches. PCRs for three endophilin-A genes were performed with respective primers: endophilin-A1: forward primer 5'CCACGAACGAACGACTCCCAC3' and reverse primers 5'-CGCACCTGCACGCGCCCTACC-3' for WT, 5'-TCATAGCCGAATAGCCTCTCC-3' for KO; endophilin-A2: forward primers 5'-CTTCTTGCCTTGCTGCCTTCCTTA-3' for WT; 5'-CCTAGGGGCTTGGGTTG-TGATGAGT-3' for KO and reverse primers 5'-GCCCCACAACCTTCTCGCTGAC-3' for WT, 5'-CGTATGCAGCCGCCGCATTGCATC-3' for KO; endophilin-A3: forward primer 5'-CTCCCCATGGTGGAAAGGTCCATTC-3' and reverse primers 5'-TGTGACAGTGGTGACCACAG-3' for WT, 5-'CAACGGACAGACGAGAG-ATTC-3' for KO. The resulting PCR products were run on a 1% agarose gel, yielding distinctive band sizes for WT and KO alleles: endophilin-A1 WT ~384 bps, KO ~950 bps; endophilin-A2 WT ~1,280 bps, KO ~1,000 bps; endophilin-A3 WT ~325 bps, KO ~465 bps. PCR products with both WT and KO bands indicate a heterozygous (HT) animal.

**Outcomes:**

To characterize gait and posture in WT mice with advancing age, we have performed kinematic gait analysis in these animals (**Figure 2**; **Movie 1**). While some parameters, for example SW (average distance between fore or hind limbs normalized to animal width; see also **Table 1**), remain unchanged in WT animals with advancing age, other parameters change progressively (**Figure 2A-C**). For instance, the hind limb double support (time relative to stance duration that both hind limbs are in contact with the ground at the same time) increases from 38% to 55% from 1 month to 18 months (**Figure 2B**). This parameter is often associated with posture instability[35]. Moreover, limb loading (maximal rate of change of the paw area in the breaking phase) increases from 38 $cm^2/s$ to 59 $cm^2/s$ from 1 month to 18 months (**Figure 2C**). Fast deceleration can be interpreted as an indicator for reduced muscle strength. The overall running ability is not affected in WT animals (94% are able to run at 30 cm/s at 18 months, **Figure 3A**). In addition to characterizing gait and posture parameters that stay unaffected, or change progressively with advancing age in WT mice, we have documented that the kinematic gait analysis using VPI is a suitable method to study the age-related mild alterations in gait and posture.

While the overall running ability is not affected in WT animals, several endophilin mutant lines show altered ability to walk or run on the motorized treadmill (**Figure 3A**), as reported in Murdoch *et al.*[13] on the smaller data set. Notably, while at 1 month of age all endophilin 1KO-2HT-3KO mice are all able to run at 30 cm/s, at 18 months of age 81% of the same animals are not able to run (**Figure 3A**, note that larger cohorts were analyzed than the ones reported previously in [13]). Interestingly, the endophilin mutants that lack fewer endophilin alleles (*i.e.,* endophilin 1KO-2HT-3WT) are also affected, but to a lower degree (**Figure 3A**).

Even though endophilin 1KO-2HT-3KO mutants show severe motor impairments with advancing age[13], several gait parameters are not changed in comparison to the WT control, also at age of 18 months. For instance, step angle variability (the standard deviation of the step angle) remains unchanged (**Figure 3B**). Notably, many other parameters, for example propel time (the percentage of stance time that the paws are in the propulsion phase), are not different at 1 month of age, but progressively become worse with aging (**Figure 3C**; see also **Movie 2**). This illustrates that both age-dependent parameters as well as the neurodegenerative mutant-specific variables can be studied with a kinematic gait analysis approach.

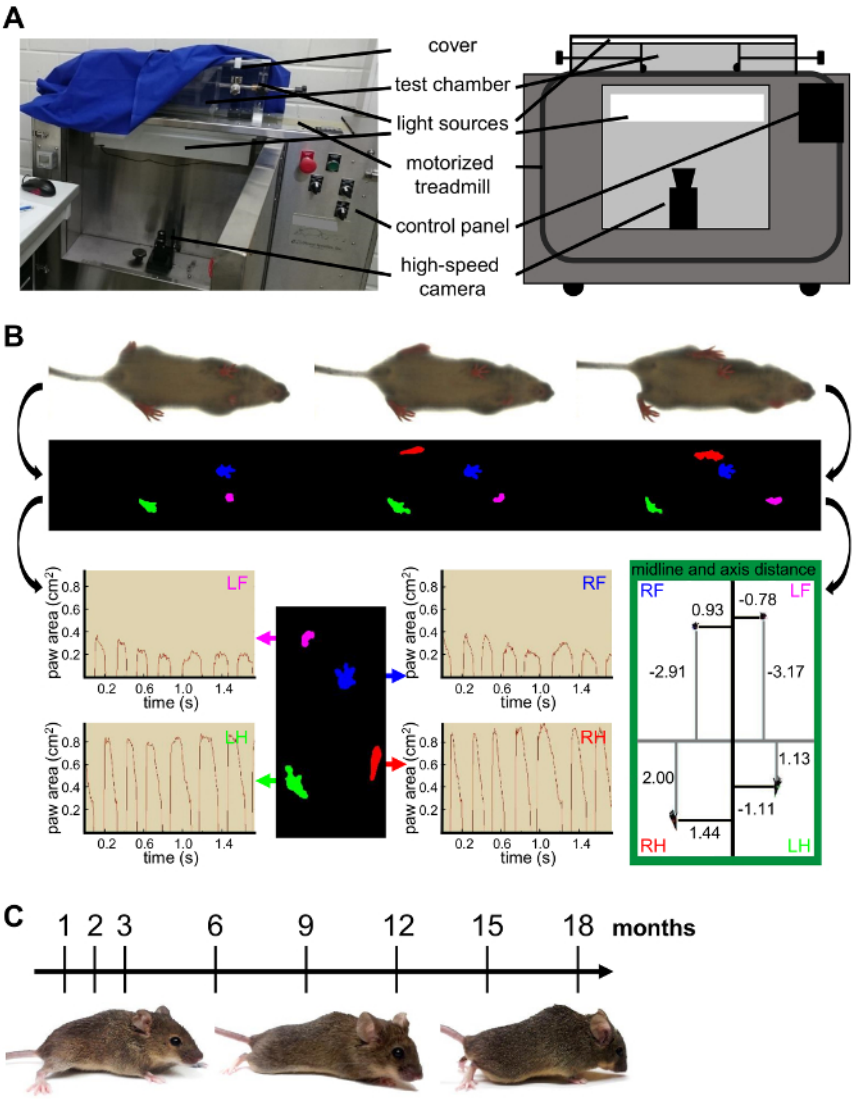



**Figure 1. Ventral plane imaging setup and principle.** (**A**) Photo and schematic drawing of a gait analysis setup. (**B**) Analysis software principle: From the recorded underside of a mouse running on a transparent treadmill, the software calculates the digital paw prints. Their dynamics during the running is measured as paw area size over time, and this is used as a basis to calculate gait rhythm and posture parameters. (**C**) Time course of the gait analysis experiment performed on endophilin mutants. The locomotion and gait were assessed at 1, 2, 3, 6, 9, 12, 15, and 18 months. Images show the endophilin 1KO-2HT-3KO mouse at 2, 12, and 18 months. Please click here to view a larger version of this figure.

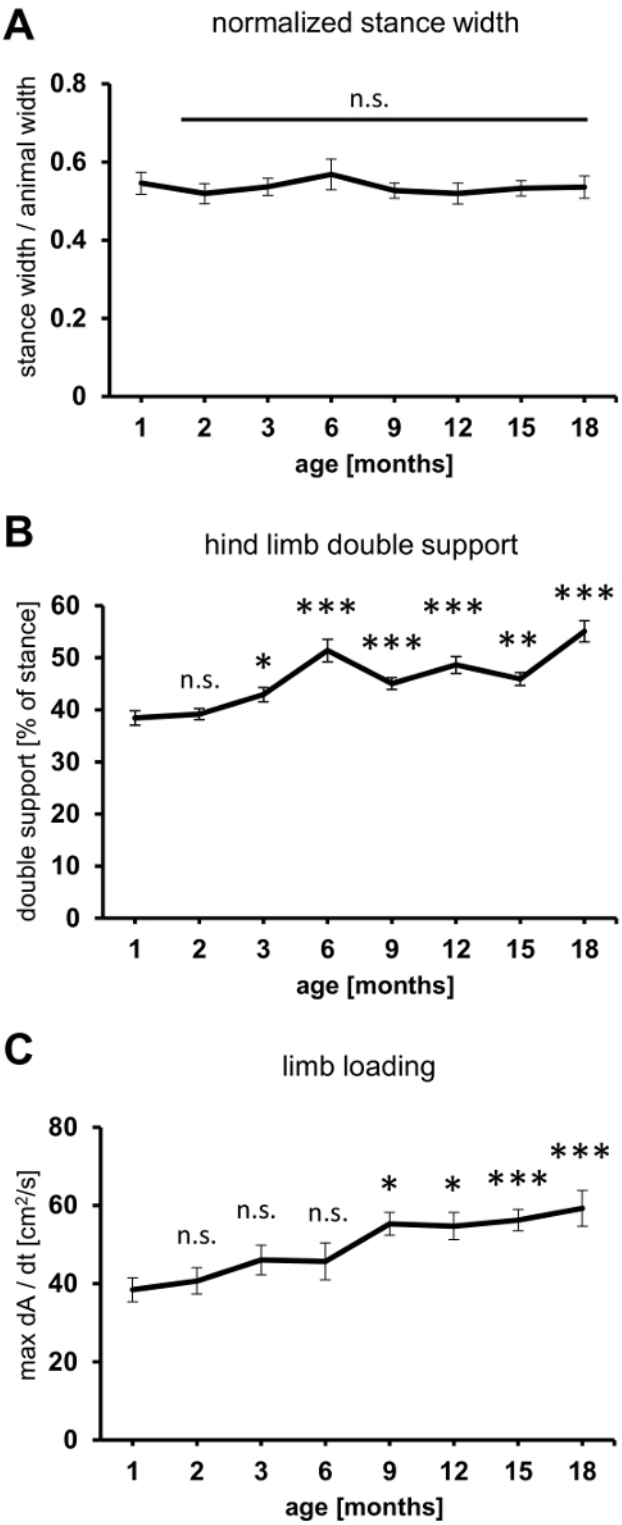



**Figure 2. Gait analysis in wild-type mice with advancing age.** The locomotion and gait in WT (C57BL/6J) mice were assessed at 1, 2, 3, 6, 9, 12, 15, and 18 months. (**A**) The stance width normalized to animal width of WT animals does not change with advancing age. (**B**) The hind limb double support increases with age in WT animals. The graph shows the percentage of stance time that both hind limbs are on the ground at the same time. An increase in this parameter reflects gait instability. (**C**) The limb loading (the maximal rate of change of the paw area in the breaking phase) increases with age in WT animals. More rapid deceleration might be an indicator for reduced muscle strength. All graphs represent mean value ± SEM; *p* values were calculated from 2-tailed *t*-tests versus the 1-month old WT, and are represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Please click here to view a larger version of this figure.

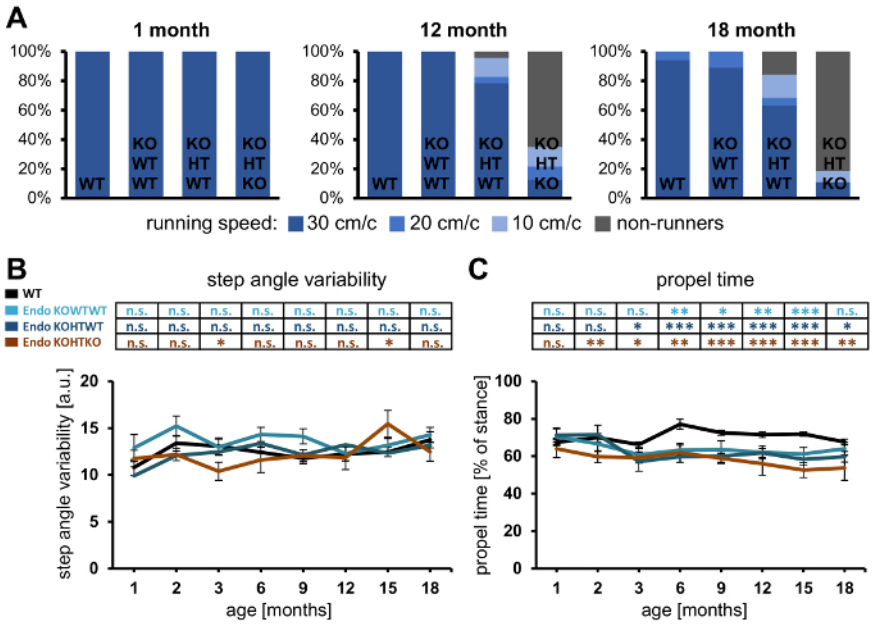
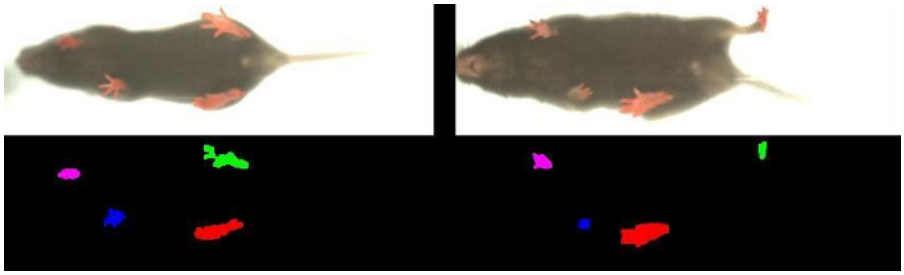
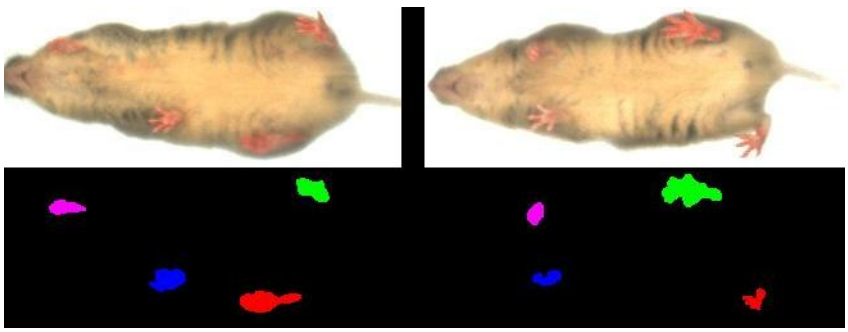



**Figure 3. Gait analysis in endophilin mutants with advancing age.** (**A**) The running speed of endophilin mutants at 1, 12, and 18 months, calculated from an expanded dataset in comparison to Murdoch *et al.*[13] Bar colors reflect the percentage of animals able to run at 30 (dark blue), 20 (blue), or 10 cm/s (light blue) on the motorized treadmill, or refuse running on the setup (grey). While all tested animals can run at 30 m/s at 1 month, the endophilin mutants develop running deficits as they age. (**B-C**) The step angle variability and propel time in WT (black), endophilin 1KO-2WT-3WT (turquois), endophilin 1KO-2HT-3WT (dark blue), and endophilin 1KO-2HT-3KO (brown) mice. The step angle variability shows no difference in aging WT animals, or between WT and endophilin mutants. The propel time (as the percentage of stance) is not significantly changed between endophilin mutants and WT at 1 month, but decreases in the endophilin mutants as the mice age. All graphs represent the mean value ± SEM; *p* values were calculated from 2-tailed *t*-tests versus age-matched WT, and are represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Please click here to view a larger version of this figure.



**Movie 1. Gait analysis in wild-type (C57BL/6J) mouse at 3 (left) and 18 months of age (right).** The original video (top) is translated to a "digital paw print" video (bottom). The video speed has been slowed down 5 times so that details can be better appreciated. At the 18-month time point, note the hesitation of the right hind paw (red in the digital paw print) at ~2 s, and of the right front paw (blue in the digital paw print) at ~4 s. The video speed has been slowed down by a factor of 10. Please click here to view this video. (Right-click to download.)



**Movie 2**. **Gait analysis in endophilin 1KO-2WT-3WT (control; left) versus endophilin 1KO-2HT-3KO (right) mice at 18 months of age.** The video speed has been slowed down by a factor of 5 so details can be better appreciated. The endophilin 1KO-2HT-3KO mouse displays gait alterations that can be seen as the less stable running of the animal. Please click here to view this video. (Right-click to download.)

## Discussion

Studying the motor coordination is a useful approach in the characterization of models of neurodegenerative diseases, especially for diseases like PD in which motor coordination is severely affected. With the help of a kinematic gait analysis functional assay, we can identify subtle changes in the gait of animals at the onset of locomotion problems, or in models with weak neurodegeneration and hence relatively modest phenotype. Given the wide range of phenotypes in various models of neurodegenerative diseases that encompasses small gait anomalies and severe movement impairments, this method is well suited to assess gait parameters based on the animal's age and ability to move. Severely impaired animals can be recorded walking at a low speed on a plane treadmill, while less impaired models can be recorded running uphill or downhill at a high speed. This can reveal gait differences between the neurodegenerative model and its littermate control without overexerting the animals.

With this protocol, we demonstrate the adequateness of the VPI method to monitor the development of motor impairments with aging in mice. Testing WT mice at multiple time points as their age advances have allowed us to identify age-dependent gait abnormalities and characterize how they progress with aging. In addition, when handling mouse models for neurodegeneration, an issue that often presents is that due to the symptoms not related to motor behavior (*e.g.,* anxiety, apathy, difficulties in learning), the willingness of the animal to perform even a simple motor task such as running, is reduced. Here, we suggest method modifications and motivational tools to encourage running on the illuminated motorized treadmill that can be helpful to successfully apply kinematic gait analysis to aging mouse lines with neurodegenerative changes. Further, we use a simple trick of applying finger paint to the animal's paws and show that it can significantly help to improve the recorded data quality. Obtaining good video recordings is the most critical step of gait analysis: the success of the analysis depends, like every automated or semi-automated analysis of images or videos, on the quality of the raw data. Low-quality videos cannot be improved at later steps in the analysis, and usually have to be excluded from the analysis process.

While systematically studying gait and posture of both WT and several endophilin mutant lines over a span of 18 months, we have noticed that even WT mice and mice with no obvious locomotion/running issues (*i.e.,* endophilin 1KO-WT-WT), show alterations in several gait and posture parameters with advancing age in a progressive way (**Figure 2** and **Figure 3A**). Interestingly, we have also noticed that while abnormalities in several gait and posture parameters observed in aging endophilin mutants develop in the same direction and slope as in the WT/control animals, others do not (**Figure 3**). Lastly, it is important to note that even if aged WT mice and young endophilin mutants do not display any obvious locomotion, gait, and posture defects when observed by eye, changes in selective gait and posture parameters can be detected with this approach.

Testing the mouse motor behavior is one of the most comprehensive ways to illustrate that a mouse model manifests major aspects of a human condition. As a result, a number of tests have been developed to assess various aspects of motor behavior. These tests include the open field test (general locomotor activity), rotarod (motor coordination, ataxia), grip strength (muscle strength), running wheel (activity), hanging wire test (endurance), ladder beam walking task (fine motor coordination, sensorimotor skill), gait analysis (locomotion, limb coordination), and others (summarized in Wahlstein[36]). The different tests have specific advantages and disadvantages and their read-outs are usually limited to the aspect (or aspects) of motor behavior that they were designed to address. For that reason, it has become common practice to perform a battery of motor behavior tests to cover the main aspects of this area.

Gait analysis is often not included in these batteries, in part due to a report by Guillot *at al.*[37], that found that gait analysis does not detect motor deficits in animal models of PD and ALS, and in part due to the laborious method and limited output. However, the Guillot *et al.* report has been challenged by research that addresses several limitations in the study design[38]. The usefulness of this method in the analysis of gait in mouse models with neurodegeneration has been demonstrated by a number of recent publications[10,11,12,39,40,41,42,43], also including our work[13].

VPI recordings come with several advantages over the conventional method of painting the paws with ink and letting the mouse run on a white sheet of paper[44]. The most obvious is the fact that with the motorized treadmill, the running speed of the animal is controlled, which has a strong influence on several gait parameters[1]. In addition, some gait abnormalities become detectable only when the animal runs at a demanding high speed and/or an incline/decline, which would not be seen in voluntary running. Furthermore, the elaborate analysis by hand is replaced by a semi-automated, high-throughput analysis. For that reason, the number of animals tested in each group can be increased, which in turn decreases the effect caused by the variability that is inevitable in living animals. In summary, we recommend that the modified version of the VPI gait analysis is included in the standard motor test batteries to complement the analysis of motor impairments in rodent models of neurodegeneration and/or aging.

## Disclosures

The authors declare no competing financial interests.

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