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TITLE:

Implantation of Osmotic Pumps and Induction of Stress to Establish a Symptomatic, Pharmacological Mouse Model for DYT/PARK-ATP1A3 Dystonia

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SUMMARY:

We provide a protocol to generate a pharmacological DYT/PARK-ATP1A3 dystonia mouse model via implantation of cannulas into basal ganglia and cerebellum connected to osmotic pumps. We describe the induction of dystonia-like movements via application of a motor challenge and the characterization of the phenotype via behavioral scoring systems.

ABSTRACT:

Genetically modified mouse models face limitations, especially when studying movement disorders, where most of the available transgenic rodent models do not present a motor phenotype resembling the clinical aspects of the human disease. Pharmacological mouse models allow for a more direct study of the pathomechanisms and their effect on the behavioral phenotype. Osmotic pumps connected to brain cannulas open up the possibility of creating pharmacological mouse models via local and chronic drug delivery. For the hereditary movement disorder of rapid-onset dystonia-parkinsonism, the loss-of-function mutation in the α3-subunit of the Na^+/K^+-ATPase can be simulated by a highly specific blockade via the glycoside ouabain. In order to locally block the α3-subunit in the basal ganglia and the cerebellum, which are the two brain structures believed to be heavily involved in the pathogenesis of rapid-onset dystonia-parkinsonism, a bilateral cannula is stereotaxically implanted into the striatum and an additional single cannula is introduced into the cerebellum. The cannulas are connected via vinyl tubing to two osmotic pumps, which are subcutaneously implanted on the back of the animals and allow for the chronic and precise delivery of ouabain. The pharmacological mouse model for rapid-onset dystonia-parkinsonism carries the additional advantage of recapitulating the clinical and pathological features of asymptomatic and symptomatic mutation carriers. Just like mutation carriers of rapid-onset dystonia parkinsonism, the ouabain-perfused mice develop dystonia-like

movements only after additional exposure to stress. We demonstrate a mild stress paradigm and introduce two modified scoring systems for the assessment of a motor phenotype.

INTRODUCTION:

The advantages of a continuous drug delivery directly into the brain are numerous. Repetitive and frequent injections, which represent an unnecessary stress factor for animals, can be avoided and a more constant intracerebral concentration of the drug can be achieved. This is especially valid when systemically administered drugs fail to easily penetrate the blood brain barrier. Moreover, chronic drug delivery via osmotic pumps allows for the localized delivery of substrates that would otherwise have system-wide side effects. The drugs can be delivered in a targeted manner to the desired brain structures, the resulting effect can thus be directly traced. This can be utilized for an array of applications, such as the study of therapeutic effects as well as the study of pathomechanisms. This last application was used in the project herein in order to create a pharmacological mouse model for dystonia.

The analysis and understanding of dystonic syndromes, which represent the third most common movement disorder, have been heavily limited by the fact that genetic animal models largely fail to reproduce the disease phenotype found in diseased human as well as the pathophysiology. This issue is not limited to dystonic syndromes, but in fact concerns many transgenic rodent models in the field of movement disorders[1,2]. The reason for the lack of a phenotype in transgenic rodent models might be based on highly effective compensatory mechanisms[3]. In the case of dystonia, the disease is characterized by involuntary muscle contractions causing twisting movements and abnormal postures[4]. The study of secondary causes (i.e., brain injury) of dystonic symptoms, has helped to identify the structures involved in the manifestation of these motor abnormalities, such as the basal ganglia[5]. Brain imaging studies of hereditary forms of dystonia have shown functional abnormalities in almost all brain regions responsible for motor control and sensorimotor integration[6,7]. However, rodent models are still needed to deepen the understanding of the neural dysfunctions on a molecular and large scale network level as well as for the development of therapeutic options. This is where pharmacological mouse models offer the possibility to replicate the clinical and pathological features of a disease in a more precise manner.

Rapid-onset dystonia-parkinsonism (DYT/PARK-ATP1A3; RDP; DYT12) is one of the hereditary forms of dystonia. It is caused by loss-of-function mutations in the *ATP1α3* gene, which encodes for the α3-subunit of the Na^+/K^+-ATPase[8]. Furthermore, it is recognized that gene mutation carriers can be free of symptoms for years before acutely developing persistent generalized dystonia and Parkinsonism after exposure to a stressful event. Indeed, the penetrance of DYT/PARK-ATP1A3 is incomplete and stressful events acting as a trigger range from physical overexertion and extreme temperatures to overconsumption of alcohol and infections[9,10]. In order to study DYT/PARK-ATP1A3 and to find potential therapeutic interventions, it has been tried numerous times to imitate the stress-dependent disease development in rodent models. However, aside from the one existing genetic DYT/PARK-ATP1A3 mouse model, where transient abnormal and convulsion-like movements were induced by hypothermia, all published genetic mouse models for DYT/PARK-ATP1A3 have failed to produce dystonic symptoms[1,11,12]. Calderon

et al. previously demonstrated that blocking the α3-subunit bilaterally in the basal ganglia and the cerebellum via the cardiac glycoside ouabain in wild type mice results in mild gait disturbance[13]. The additional exposure to electrical foot shocks in a warm environment led to a dystonic and bradykinetic phenotype, thus demonstrating that chronic and targeted perfusion of ouabain followed by stress successfully imitates the DYT/PARK-ATP1A3 phenotype.

However, exposing animals to electrical foot shocks in a warm environment of 38-40 °C over a two hour-period induces pain and anxiety in animals, which represent confounding factors, especially for the assessment of changes in the catecholamine system related to the development of dystonia. Thus, we herein describe a different kind of stress paradigm with high translational value, which relates back to the fact that mild to moderate exercise have been described as triggers in DYT/PARK-ATP1A3 patients[9]. Moreover, repetitive exercise is a well-known trigger for focal dystonia[14]. Mice were repeatedly subjected to challenging motor tasks comprised of three descends of a wooden pole ("pole test") and three runs on a Rotarod apparatus ("Rotarod performance test"). The placement of animals on the top of a 50 cm wooden pole was used to coerce the animals to descend, the Rotarod apparatus was employed to subject mice to forced activity by placing them on a rotating rod.

The characterization of the motor phenotype of a mouse model for dystonia is particular challenging due to the lack of predefined tests and scores. However, one variation of a motor disability assessment has been repeatedly used over the last years in order to evaluate the severity and the distribution of dystonia-like movements in rodents[13,15,16]. We herein present a modified version of the dystonia rating scale, which proved to be effective in evaluating the dystonia-like phenotype of animals when observed over a time period of four minutes. As a second method of assessing dystonia-like movements, we present a newly-developed scoring system for the assessment of abnormal movements during a tail suspension test. It allows for the assessment of the frequency and duration of dystonia-like movements and postures of the front limbs, hindlimbs as well as trunk.

PROTOCOL:

All procedures were performed in accordance with applicable international, national, and/or institutional guidelines for care and use of animals. The local authorities at the Regierung von Unterfranken, Würzburg, Germany, approved all animal experiments.

1. Priming of osmotic pumps

NOTE: This step has to be performed at least 48 h prior to surgery. ALZET osmotic pumps need to be prefilled in order to ensure that the pumping rate reaches a steady state before implantation.

1.1. Prepare the desired solution for chronic perfusion beforehand. Ensure that the solvent and agent are compatible with the osmotic pumps and catheters. For the project herein, thaw a 10x stock solution of ouabain (stored at -20 °C) at room temperature and vortex for 10 s.

CAUTION: Ouabain is toxic: it should be handled only with gloves and opened only under a sterile hood to avoid inhalation.

1.2. Turn the sterile hood on; disinfect the hood as well as all the instruments and material needed before putting them under the hood.

1.3. Put on surgical gloves before handling the osmotic pumps. Separately prepare the ouabain-solution designated for the pumps of the striatum and the cerebellum.

NOTE: Consider that the concentration of the solution in the pump for the striatum has to be doubled compared to the concentration of the solution in the pump designated for the cerebellum. This is due to the fact that the pump for the striatum is connected to a double cannula, the flow rate, however, is the same as for the pump of the cerebellum, which is connected to a single cannula.

1.4. Calculate the amount of solution needed using the following formula:

mass delivery rate (k_o) = volume delivery rate (Q) x concentration of the agent in the vehicle (C_d)

For the present project, the osmotic pumps were primed and filled with either ouabain solution at a concentration of 11.2 ng/h or 0.9% saline for the control group.

1.5. Vortex both ouabain solutions for 10 s and sterile filter (i.e., 0.22 µm syringe-end filter) them into separate, new microcentrifuge tubes, using a different filter for each solution.

1.6. Weigh the empty osmotic pumps together with the flow moderator (about 0.4 g).

1.7. Fill a 1 mL syringe with a 27 G filling cannula (preferably with a blunt tip) with sterile filtered ouabain-solution or vehicle solution.

1.8. Get all the air bubbles out of the syringe, hold the osmotic pump in an upright position, insert the cannula all the way into the pump and slowly fill the reservoir until excess solution appears on top (avoid rapid filling as this can lead to air bubbles in the pump). Next, insert the flow moderator into the pump (excess solution should appear on top).

1.9. Pull the flow moderator out (about 5 mm), carefully take off the white flange with scissors and connect a piece of vinyl tubing to the flow moderator. Ensure that the catheter has a length of approximately 2 cm to allow proper mobility of the animal.

1.10. Weigh the filled osmotic pump once more to ensure that the difference in weight between filled and empty pump is concordant with the expected weight of the solution loaded.

NOTE: For most aqueous solutions this weight is equal to the volume in microliters. If the weight

does not correspond to the expected volume, air might be trapped inside the pump, which needs to be emptied and refilled.

1.11. For the priming of the pumps, prepare two 2 mL microcentrifuge tubes, one tube for the striatum and one for the cerebellum.

1.12. Submerge the pumps into the microcentrifuge tubes filled halfway with 0.9% saline solution. Do not submerge the open end of the catheter. Put the microcentrifuge tubes into a thermocycler for 48 h at 37 °C in order to ensure that the pump has reached a steady pumping rate before implantation and the catheters are prefilled.

2. Cannula and osmotic pump implantation

2.1. Place the mouse (male, C57Bl/6N, 11-12 weeks of age) into a chamber designed for inhalation anesthesia; set the flow rate of isoflurane to 2-3% and the flow rate of oxygen to 2 L/min.

2.2. After the mouse is deeply anaesthetized according to approved protocols, shave the top of the animal's head, dorsal neck and proximal third of the back.

2.3. Place the animal into a stereotactic frame and continue the anesthesia with isoflurane via a mouse anesthesia mask designed for the stereotactic instrument (isoflurane flow rate 1.5-2%, 2 L/min oxygen).

2.4. Using rubber tips or non-rupture ear bars, fix the head of the animal in the stereotactic frame, taking care that the head is leveled.

2.5. In order to prevent hypothermia, place a heating pad under the animal, insert a rectal temperature probe and set the temperature to 37 °C. Protect the eyes of the animal from drying out by using a drop of ophthalmic ointment on each eye.

2.6. Apply an analgesic, such as Carprofen (5 mg/kg bodyweight), subcutaneously before beginning surgery.

2.7. With a syringe subcutaneously inject up to 0.5 mL of bupivacaine 0.25% and wait 30 s for the local anesthesia to take effect.

2.8. Disinfect the shaved areas thoroughly with an antiseptic, such as octenidine dihydrochloride.

2.9. After thorough disinfection of the surgical area, use a scalpel to place an incision at the top of the head and use scissors to continue the incision down to the fore limbs. Expose the skull with the help of bulldog clamps and wipe the periosteum with a sterile cotton-tipped applicator.

2.10. Align either a pen or the tip of a cannula stained in black ink with bregma and use the

appropriate coordinates to mark the three entry points for the brain cannulas on the skull (coordinates for the bilateral holes needed for perfusion of the basal ganglia: anterior/posterior: + 0.74 mm, medial/lateral: +/- 1.50 mm (+ indicating the right side, - the left side relative to bregma); coordinates for the hole at the midline of the cerebellum: anterior/posterior: - 6.90 mm). Next, carefully drill the holes for the double cannula designated for the basal ganglia and the single cannula designated for the cerebellum (**Figure 1A**).

2.11. Drill a fourth hole for a small screw in-between the striatum and the cerebellum. This screw will eventually be embedded in dental cement and provide additional hold for the cannulas. Using fine forceps and a screw driver, carefully introduce the screw into the small hole until it is firmly fixed in the skull. Do not implant the screw too deeply as this damages the brain tissue.

NOTE: It is recommended to continue with the osmotic pump implantation before inserting the osmotic cannulas. This avoids causing any accidental damage to the cannulas when implanting the pumps.

2.12. In order to create a small pocket for the osmotic pump on each side of the animals back, use tissue forceps to separate the subcutaneous tissue layers. Advance the forceps towards one hind leg and open the forceps slightly in order to widen the subcutaneous pocket. Repeat the same procedure for the other side, first removing the forceps from the incision and then pushing them gently towards the second hind leg.

NOTE: The pocket should permit the pump to easily slide in, however, it should not have too much room to move under the skin.

2.13. With the help of tissue forceps take the first pump together with the connected piece of tubing and slide it into one subcutaneous pocket. Repeat the same procedure with the second pump.

2.14 Using a minipump holder, introduce a single osmotic cannula with a custom length of 3.0 mm into the hole drilled in the midline of the cerebellum. Detach the cannula head carefully and fix the cannula as well as the small screw with dental cement, taking care as not to cover the connecting piece for the tubing of the osmotic pump. Ensure that the dental cement surrounding the cannula has fully dried before continuing with surgery.

2.15. Before inserting a double cannula with a center-to-center distance of 3.0 mm and custom-made length of 4.0 mm into the bilaterally drilled holes above the basal ganglia, attach two short pieces of vinyl tubing (0.5 cm) to the two connecting pieces of the double cannula. Connect the vinyl tubings with a bifurcation adaptor and carefully prefill the entire tubing system including the adaptor and cannula with ouabain solution or vehicle (room temperature, sterile filtered beforehand). This can be done best with a 1 mL syringe and a 27 G filling cannula introduced into the single rear end of the bifurcation adaptor (**Figure 1B**).

2.16. Using a minipump holder, carefully insert the double cannula into the bilateral holes. Use a

clamp to detach the cannula head and fix the double cannula with dental cement.

2.18. Connect the catheters of the osmotic pumps to the bifurcation adaptor as well as the single cannula, respectively. Ensure that the catheters have a strong hold on the connecting pieces of the cannulas.

NOTE: Both pumps have an equal flow rate, so be careful to connect the osmotic pump with the double concentrated solution to the bifurcation adaptor to ensure that the same concentration reaches both the basal ganglia and the cerebellum.

2.19. Close the incision on the back of the animals with stitches as far as possible in direction of the skull, being careful not to overstretch the skin (**Figure 1C**).

2.20. Subcutaneously inject 0.5 mL of 0.9% saline, which should have body temperature, into a skin fold on each side of the back of the animals in order to avoid dehydration of the mice, carefully avoiding the pumps.

3. Motor challenge

3.1. Subject ouabain- or vehicle-perfused mice to challenging motor tasks as a form of mild stress exposure 4 h post-surgery and repetitively every 24 h afterwards in order to induce dystonia-like movements. This does not include a behavioral characterization; its purpose is to induce a higher stress level compared to normal cage keeping.

3.2. Place the mouse onto a 50 cm rough-surfaced, wooden pole with a diameter of 1 cm, nose facing downwards. Ensure that the pole is placed into a large cage with enough bedding in case of falls. It is not necessary to measure the time of descent, but look out for involuntary hyperextensions of front limbs and hindlimbs presented by ouabain-perfused animals while descending the pole. Let mice descend the pole three times and allow 2 min of recovery between descends.

NOTE: Ouabain-perfused mice should present first symptoms like bradykinesia 4 h post surgery. However, 24 h after surgery mice should start to present involuntary hyperextensions of front limbs and hindlimbs as a sign of dystonia-like movements during descend.

3.3. For the second motor task, place mice on the rotating rod as done for the Rotarod performance test. The Rotarod apparatus is not used as a measure of the latency to fall, the aim is to subject mice to forced activity. Place the mice on the rotating rod three times and allow 2 min of recovery between tests.

NOTE: To increase stress exposure, use an accelerating Rotarod apparatus. For the project described herein, the rod accelerates from 5 to 50 rpm over a set time period of 300 sec.

3.4. Let animals recover for 30 min in between the pole test and the Rotarod performance test

and again another 30 min before scoring for dystonia-like movements as described under protocol step 4. In between the repetitive stress exposures, allow mice to recover for 24 h-intervals.

4. Scoring systems for the assessment of dystonia-like movements

NOTE: The experimenter should be blinded to the group assignment analyzed to prevent bias. The behavioral tests used to characterize the phenotype of the mice are two scoring systems: a dystonia rating scale scoring abnormal, dystonia-like movements and a behavioral score using the tail suspension test. Assess the dystonia-like movements after a recovery time of 30 min following the exposure to mild stress.

4.1. Dystonia rating scale

NOTE: Due to the lack of predefined behavioral tasks, the dystonia rating scale was established as an observer-based scoring system similar to the clinical rating scales of human dystonia. It is a modified version of the dystonia rating scale used by Calderon et al.[13].

4.1.1. Record posture and gait of animals over a period of 4 min after animals have been placed in a plastic or wooden box.

4.1.2. Score for frequency and distribution of dystonia-like movements from 0 to 4 points: (0) normal motor behavior; (1) abnormal motor behavior, no dystonia-like movements; (2) mild motor impairment with mild focal dystonia-like movements; (3) moderate motor impairment with severe focal dystonia-like movements; (4) severe impairment, with sustained, generalized dystonia-like movements (**Figure 2**). Consider the following movements or postures as dystonia-like: hyperextension of front limbs, wide stance or hyperextension of hindlimbs as well as kyphosis. Consider dystonia as focal in case a single body part is affected and as generalized in case the trunk and at least two other body parts are affected.

4.2. Tail suspension test

NOTE: The tail suspension test is often used to observe and score for hindlimb clasping. This is however a highly unspecific phenotype indicating a motor impairment. The following protocol proposes a scoring system specific for dystonia-like movements. Due to the generalization of dystonia in DYT/PARK-ATP1A3 patients, dystonia-like movements should be assessed in front limbs, trunk and hindlimbs. A newly-developed scoring system from 0-8 points was developed, a total score < 2 indicated that no dystonia-like movements were present (**Figure 3**).

4.2.1. Pick the mouse up by the tail near its base and lift the animal up. Record a 2 min video of the tail suspension test and assign a score in a subsequent, thorough analysis of the recording.

4.2.2. Score the front limbs from 0 to 4 points, where repeated or sustained tonic retractions of one or both front limbs, as well as a hyperextension combined with crossing of the front limbs,

were classified as dystonia-like: (0) no abnormal movements; (1) reduced movement of front limbs with hyperextension of paws seen ≥ 50% of the recorded time; (2) mild dystonia-like movements of front limb(s) < 50% of the recorded time; (3) mild dystonia-like movements of front limb(s) ≥ 50% of the recorded time or severe < 50% of the recorded time; (4) severe dystonia-like movements of front limb(s) ≥ 50% of the recorded time.

NOTE: Hindlimb clasping is an abnormal movement that should not be scored as dystonia-like.

4.2.3. Score the hindlimbs from 0 to 3 points, where retraction and clenching of rear limbs as well as sustained hyperextension were assessed as dystonia-like: (0) no abnormal movements; (1) reduced movement of hindlimbs with hyperextension of paws seen ≥ 50% of the recorded time; (2) dystonia-like movements of one hindlimb; (3) dystonia-like movements of both hindlimbs.

4.2.4. In case of truncal distortion > 80% of the recorded time, an additional point is added to the score.

4.2.5. Place the animal back into its cage.

REPRESENTATIVE RESULTS:

Figure 4 has been modified from Rauschenberger et al.[17]. For data analysis of both the dystonia rating scale (**A**) and the tail suspension test (**B**), calculate the total score for each time point for each animal. The mean of each time point and each group should be plotted on an appropriate graph. The distribution of the values should be investigated and the appropriate statistical test should be applied to determine significance. With a sufficient number of animals, a motor phenotype can be detected both with the dystonia rating scale as well as with the assessment of abnormal movements in the tail suspension test. The dystonia-like phenotype is demonstrated by the significantly higher motor score in both assessments in the ouabain-perfused, stressed group compared to ouabain-perfused, non-stressed mice as well as the control mice.

FIGURE AND TABLE LEGENDS:

Figure 1: **The main surgical steps for cannula and osmotic pump implantation.** (**A**) For the indicated coordinates, holes need to be drilled bilaterally for the double cannula designated for the basal ganglia and for the single cannula placed at the midline of the cerebellum. The two fully-constructed osmotic pumps are shown on each side of the animal. (**B**) The picture shows an implanted, single cannula into the cerebellum, fixed with dental cement. The double cannula for the basal ganglia should be connected to the bifurcation adaptor and prefilled with ouabain before implantation. (**C**) Image of the finished procedure.

Figure 2: Assessment of dystonia-like movements with a dystonia rating scale. During a 4 min video, dystonia-like movements were scored based on body distribution and duration. Involuntary hyperextension of the front limbs, a wide stance or hyperextension of hindlimbs as well as kyphosis were rated as dystonia-like.

Figure 3: **Assessment of dystonia-like movements during a tail suspension test.** The newly-

developed scoring system for abnormal movements during a 2 min tail suspension test evaluates dystonia-like movements in front limbs, hindlimbs and trunk from 0-8 points in total. For the front limbs, a hyperextension and crossing of the front limbs as well as a tonic flexion towards the trunk qualified as dystonia-like. For the hindlimbs the involuntary hyperextension as well as retraction with extension over the midline was scored as dystonia-like. A truncal distortion over 80% of the recorded time was scored with one point.

Figure 4: **Representative graphs of the dystonia-rating scale and the tail suspension test.** (**A**) The graph depicts the dystonia-rating scale for NaCl-perfused, stressed mice (dotted black line), ouabain-perfused, non-stressed mice (dotted orange line) and ouabain-perfused, stressed mice (dark blue line). For each time point, the mean values ± standard error of the mean (SEM) are shown. (**B**) The diagram shows the assessment of abnormal movements during a 2-min tail suspension test for NaCl-perfused, stressed mice (dotted black line), ouabain-perfused, non-stressed mice (dotted orange line) and ouabain-perfused, stressed mice (dark blue line). Statistical analysis was done for the dystonia rating scale and the tail suspension test scoring using the two-tailed Mann-Whitney test. Bonferroni-Holm correction (§) of the p-values showed a significant difference for the observational period of 72 h. Dark blue * denote significant differences between ouabain-perfused, stressed mice and ouabain-perfused, non-stressed mice mice, black * denote significant differences between NaCl-perfused, stressed mice and ouabain-perfused, stressed mice as well as between NaCl-perfused, stressed mice and ouabain-perfused, non-stressed mice.

DISCUSSION:

This DYT/PARK-ATP1A3 pharmacological mouse model allows for the detailed analysis of intracerebral structural and neurochemical changes induced solely by inhibition of the sodium-potassium ion pump in the basal ganglia and cerebellum as well as alterations related to stress exposure. In case of mice, a maximum of two osmotic pumps can be subcutaneously implanted. We herein present a method detailing chronic drug delivery to multiple brain structures by implementing a double cannula connected to a bifurcation adaptor in addition to a single cannula. This methodology can be used for any application requiring multiple brain structures to be perfused simultaneously and chronically.

We present a mouse model of a rare movement disorder, where patients develop permanent symptoms after exposure to stress. This presumed gene-environmental interaction is still not well understood, but might represent one of the key pathomechanisms in DYT/PARK-ATP1A3 development. Different methods of exposing mice to stress have been published in the past and include electric foot shocks, restraining, cold or warm environment and exposure to various odors[11-13]. In an effort to expose mice to a mild stress factor with translational value, we herein describe the repetitive subjection of mice to challenging motor tasks. For the pole test, ouabain-perfused animals revealed involuntary hyperextension of front limbs and hindlimbs. These movements were very similar to the dystonia-like movements observed during the 4-min video recording of the animals as well as the tail suspension test. The application of mild stress in form of challenging motor tasks might prove useful in other mouse models showing motor symptoms or neurodegeneration, where gene-environmental interactions massively influence the degree

of disease progression.

There is a general lack of predefined behavioural tasks as well as rating scales to classify abnormal movements and postures in mice. Most of the available motor tasks reveal unspecific abnormalities, such as hindlimb clasping, which is a well-known phenomenon in many mouse models of movement disorders with neurodegeneration[18,19]. For the proper characterization of a phenotype, it is however necessary to analyze whether the mouse model recapitulates the salient features of the disease. Herein, we present the modified version of a dystonia rating scale used previously for the assessment of motor disability in dystonia mouse models[15,16]. We additionally developed an observer-based scoring system for the tail suspension test, which was established similarly to the clinical rating scales of human dystonia. Both rating scales show a significantly higher score in ouabain-perfused, stressed mice compared to ouabain-perfused, non-stressed animals as well as vehicle-perfused animals. The drawbacks of any observer-based scoring system are the necessary training of raters to ensure consistent scoring and to reduce observer variability as well as the danger of a possible bias of the rater if not fully blinded to the group analysed. However, observer-based scoring systems still present an easily accessible method to characterize a phenotype and can be adapted to the mouse model analyzed, as done in the present project for the assessment of dystonia-like movements. To ensure consistent scoring among different raters, training videos should be made available. To reduce any potential bias, it is recommended that different raters score the same video clips and that the individual scores are averaged. Both scoring systems mentioned within this work record the presence of dystonia-like movements in animals. The rating scales can be adapted according to the specific requirements within a project, as done previously by Ip et al., where solely the hindlimbs were scored for dystonia-like movements in a mouse model for dystonia 1 (DYT-TOR1A)[20]. The rating scales can be complemented by other previously published scoring systems, assessing for example the degree of bradykinesia in rodents as done with the locomotion disability score by Calderon et al.[13].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

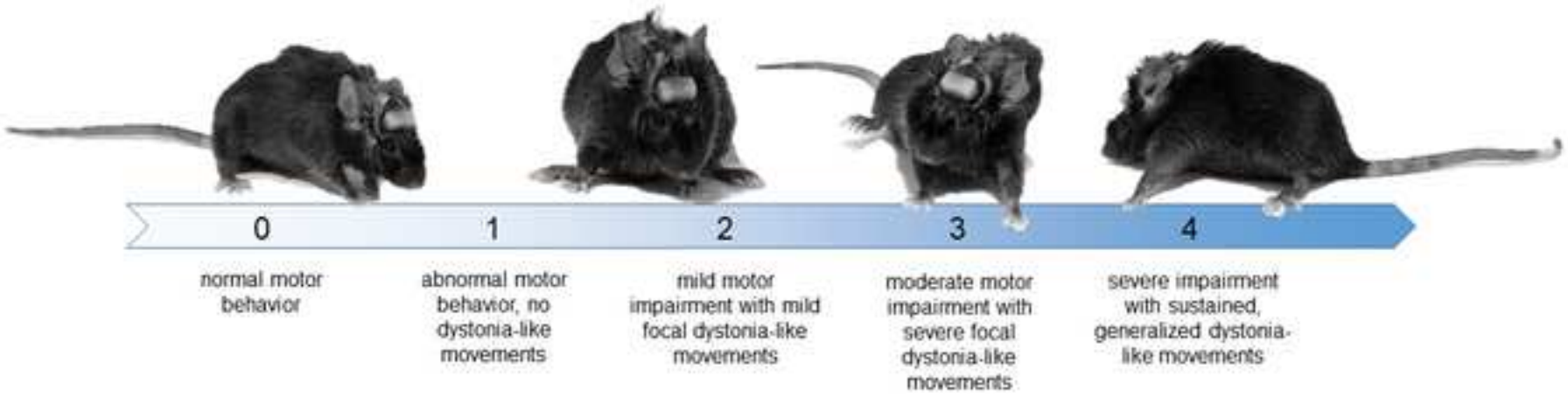

Figure 3

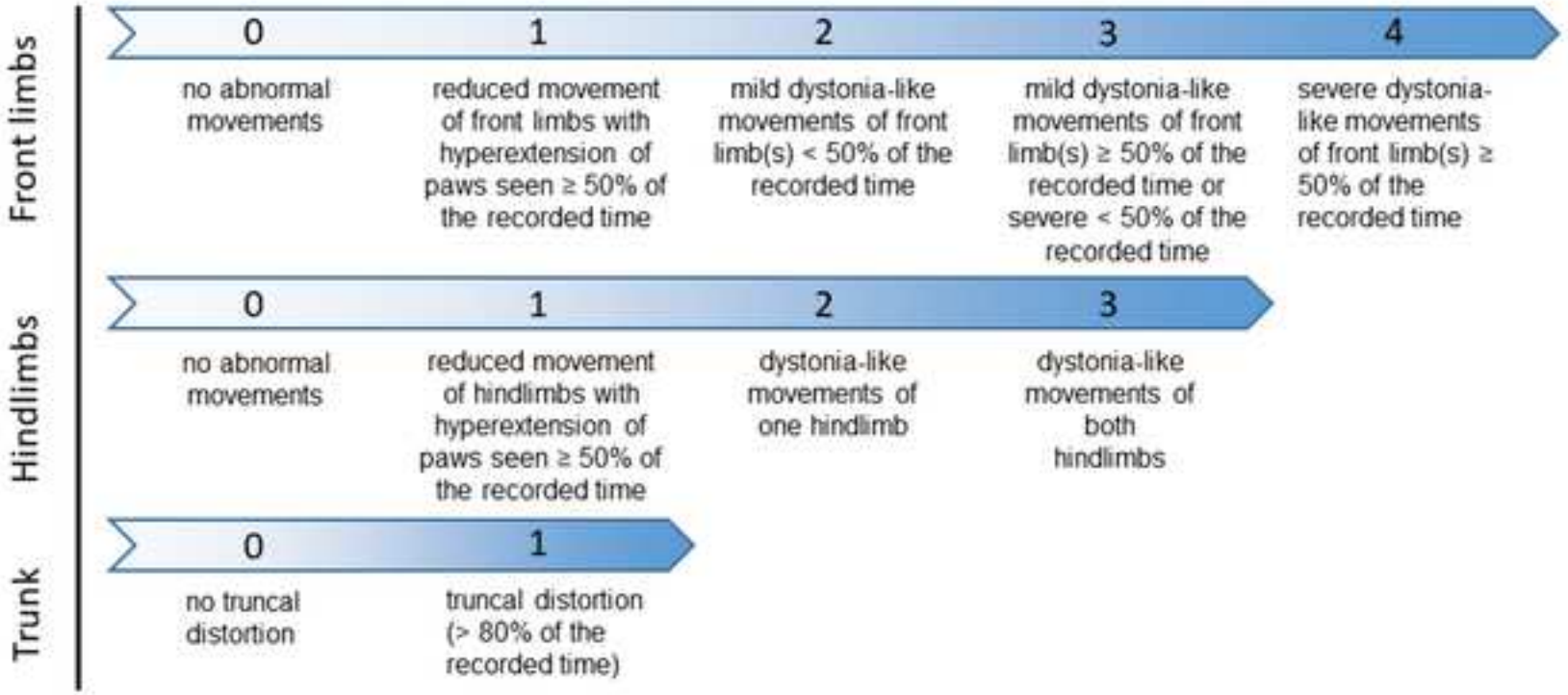

Figure 4

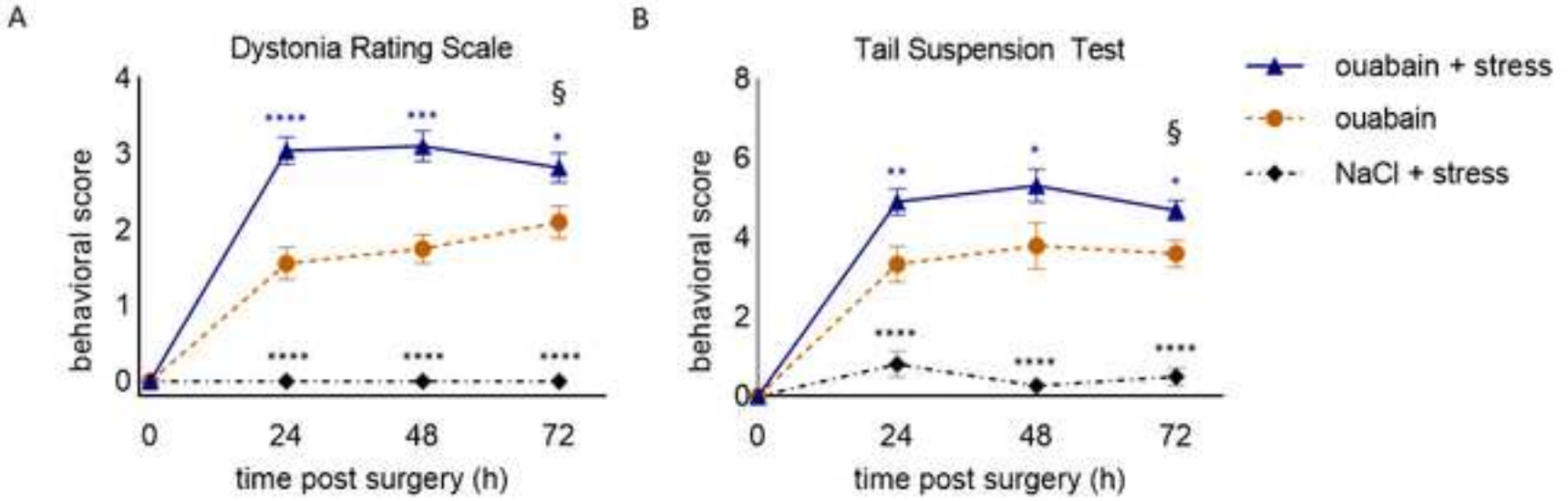

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.9% saline	Fresenius Kabi		PZN06178437
Alzet osmotic pumps	Durect	4317	model 1002, flowrate 0.25 µL/h
Anchor Screws	AgnTho's	MCS1x2	2 mm long with a thread of 1mm O.D.
Bulldog Clamps	Agntho's	13-320-035	straight, 3.5 cm
Bupivacain 0.25% Jenapharm	mibe GmbH Arzneimittel		
Cannula and Minipump Holder	Stoelting	51636	designed to hold 3.4 mm cannula heads
Cannula Bifurcation	Plastics One	21Y	custom made
Cannula tubing	Plastics One	C312VT/PKG	vinyl, 0.69 mm x 1.14 mm
Dumont #5SF forceps	Fine Science Tools	11252-00	fine forceps
eye cream Bepanthen	Bayer Vital GmbH		
Gas Anesthesia Mask for Stereotaxic, Mouse	Stoelting	56109M	
Hardened fine scissors	Fine Science Tools	14090-09	
High Speed Rotary Micromotor Kit	Foredom	K.1070-2	
Isoflurane CP 1 mL/mL, 250 mL	cp-pharma	1214	prescription needed
Isoflurane System Dräger Vapor 19.3	Dr. Wilfried Müller GmbH		
Kallocryl A/C	Speiko	1615	dental cement, liquid
Kallocryl CPGM rot	Speiko	1692	dental cement, red powder
Mouse and neonates adaptor	Stoelting Co.	51625	adaptor for mice for a traditional U-frame
needle holder	KLS Martin Group	20-526-14	
Non-Rupture Ear Bars and Rubber Tips f/ Mouse Stereotaxic	Stoelting Co.	51649	
Octenisept	Schülke	118211	
Osmotic Pump Connector Cannula for Mice, double	Plastics One	3280PD-3.0/SPC	28 Gauge, length 4.0 mm, c/c distance 3.0 mm
Osmotic Pump Connector Cannula for Mice, single	Plastics One	3280PM/SPC	28, Gauge, custom length 3.0 mm
Ouabain octahydrate 250 mg	Sigma-Aldrich	03125-250MG	CAUTION: toxic

Precision balance	Kern & Sohn	PFB 6000-1	
Rectal Thermal Probe	Stoelting	50304	
Rimadyl 50 mg/mL, injectable	Zoetis		Carprofen, prescription needed
Rodent Warmer X1 with Mouse Heating Pad	Stoelting	53800M	
RotaRod Advanced	TSE Systems		
screw driver set	Agntho's	30090-6	
Stainless Steel Burrs	Agntho's	HM71009	0.9 mm Ø burr
Stainless Steel Burrs	Agntho's	HM71014	1.4 mm Ø burr
StereoDrive	Neurostar		software
Stereotaxic instrument	Stoelting Co.		custom made by Neurostar
Stereotaxic robot	Neurostar		
suture: coated vicryl, polyglatin 910	Ethicon	V797D	
ThermoMixer C	Eppendorf AG	5382000015	

To Nam Nguyen, PhD
Manager of Review
JoVE

Würzburg, 8th of July 2020

Dear Dr. Nguyen,

We are grateful for your editorial help and the constructive criticism of the expert reviewers. In our opinion, the comments have helped to greatly improve the revised version of the manuscript: "Implantation of osmotic pumps and induction of stress to establish a symptomatic, pharmacological mouse model for DYT/PARK-ATP1A3 dystonia." We have included changes made to the manuscript (highlighted in green). Upon the editor's recommendation, we also revised the protocol section of the manuscript to better match the narration in the video (highlighted in blue), and vice versa the video narration was adapted for some parts of the protocol (time points indicated below). In addition, we would like to reply to the editor's and reviewers' comments (the editor's and the reviewers' comments are in *italic*) as follows:

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We performed a proofreading of the manuscript and corrected any spelling and grammar issues. The yellow highlight used as marking of the parts of the protocol used for filming was removed.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We revised any parts of the protocol section not written in the imperative tense (protocol steps revised: 1.1.; 1.4.; 1.9.; 1.10.; 1.12.; 2.6.; 2.7.; 2.11.; 2.12.; 3.1.; 3.2; 3.3.; 3.4.; 4.1.1.; 4.1.2.; 4.2.1; 4.2.3.).

3. Please do not abbreviate journal titles in the references.

We now included the full name of the journal titles in the references.

Changes to be made by the Author(s) regarding the video:

1. Please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol. Alternatively, the written protocol can be rewritten to match the narration.

The narration of the video was partly revised (02:00; 02:51; 03:49) and the video edited at two time points to better match the written protocol (02:18; 03:05). For other parts the protocol was

revised in order to match the narration (text highlighted in blue). The adaption of the written protocol included only minor changes, which did not change any relevant parts about the overall procedure.

2. Delivery & A/V Specifications

• Videos larger than 1920x1080 will be downconverted. Consider exporting your next revision at 1920x1080 for smaller delivery file size.

For the revision, the video was exported at 1920x1080.

• 01:18 There is "letter boxing" here that should be removed. Scale the video up, the left and right sides can be sacrificed here.

The “letter boxing” at 01:18 was removed.

• Audio levels: Introduction and Conclusion segments with the on-screen speakers are quieter than the narration. The narration may be a little too loud. Please do a round of audio mixing to make the audio levels similar overall throughout the video. The audio level peaks should fall between -12 and -6 dB.

Audio levels were edited and audio level peaks now fall between -12 and -6db throughout the video.

3. Format & Content Standards

• Title card: Consider placing Jens Volkmann and Chi Wang Ip on the same line, it may be a little easier to read.

We changed the title card as recommended, placing Jens Volkmann and Chi Wang Ip on the same line. Accordingly, we also changed this on the title card at the end of the video. Due to the changes necessary to the title upon the expert reviewers’ recommendation, the title was also revised on the title cards of the video.

• 00:29 During the transition from Lisa Rauschenberger to Susan Knorr, there is a little bit of Knorr talking to the camera before she delivers her line. See if this can be edited out.

The transition from Lisa Rauschenberger to Susanne Knorr was edited as recommended.

• 03:57 There is puff of air blown on the microphone heard on the audio track here. Can it be edited out?

The puff of air was removed from the audio track (corresponding to 04:18 in the edited video).

• 09:46 Consider placing a chapter title card for the Conclusion here.

As suggested by the editor, we added a chapter title card at the indicated time point.

Reviewers' comments:

Reviewer #1:

Manuscript Summary: Genetic animal models of movement disorders largely fail to reproduce the behavioral phenotype as well as the pathophysiology of the disease. On the contrary, pharmacological rodent models offer the possibility to study directly the pathomechanisms and the resulting effects of the behavioral phenotype. Here the Authors, in order to study DYT/PARK-ATP1A3 (DYT 12), developed an ouabain-based pharmacological animal model: osmotic pumps connected to brain cannulas, stereotaxically implanted into the striatum (bilateral cannulas) and in the cerebellum (single cannula), allow for the chronic and local delivery of ouabain. Further, mice were subjected to challenging motor tasks, as stress paradigm to trigger motor impairment and dystonia phenotype. Overall, the study is well designed and methods are clearly described. More specifically, the steps listed in the procedure are detailed and support the described outcome.

Minor Concerns: Nevertheless, some additional information would be useful, listed below:

** What type of rotarod test did the Authors use? Set-speed rotarod or accelerating rotarod test? If they used an accelerating rotarod, they should indicate the rate of acceleration.*

We thank the reviewer for this comment, an accelerating rotarod test was used for the stress paradigm. The rod accelerated from 5 to 50 rpm over a set time period of 300 sec. We added this information to the manuscript as a "Note" (line 303).

** The Authors indicate the time of recovery between three tasks of single motor test (pole test and rotarod test), but they should also indicate time of recovery between tests.*

Upon suggestion of the reviewer, we added the recommended recovery time of 30 min in-between the pole test and Rotarod performance test (line 306).

** It is not clear when, after stress paradigm, the Authors did the assessment of motor phenotype. Did they record posture and gait, with dystonia rating scale and tail suspension test, immediately after motor tests?*

We understand that this caused confusion, indeed the assessment of the motor phenotype was done 30 min after exposing the animals to motor stress. We added this information to the manuscript (line 306 and line 316).

** It could be useful for naive experimenters if Authors indicate what kind of movements are considered abnormal but not dystonia-like movements.*

We thank the reviewer for this valuable comment. The most commonly observed abnormal movement in mice is indeed hindlimb clasping, which we briefly mentioned within the protocol as well as discussion. We added a note to the scoring of dystonia-like movements of hindlimbs within the tail suspension test, detailing hindlimb clasping as an abnormal movement the experimenter should be careful not to confuse with dystonia (line 358). No other abnormal movements, that were not classified as dystonia-like, were observed.

Reviewer #2:

Manuscript Summary: This manuscript on the detailed methods of developing a pharmacological mouse model of DYT12 is a timely, significant contribution to the research on the pathomechanisms and animal model of dystonia. The methods were documented in sufficient detail for other researchers to develop similar models with other types of dystonia or movement disorders in general. The impact of the manuscript is excellent. There are several minor deficiencies that will need to be addressed.

Minor Concerns: At several places, the authors used motor stress. It is perhaps an ill-defined term. It is also possible the mice had undergone mental stress while doing the pole and rotarod tests repeatedly.

We thank the reviewer for their input, indeed an additional mental stress cannot be excluded in mice exposed to challenging motor tasks. We propose using the term "motor challenge" instead of "motor stress" and accordingly performed the necessary replacements within the manuscript. At times, such as in the title of the manuscript, the term "motor stress" was shortened to "stress".

Figure 4, it is not clear what the significance signs mean. Were the significances between owabain+stree compared with ouabain only or the saline plus stress? The authors need to redesign the figure or state them clearly in the figure legends.

We apologize for this confusion; indeed, the significances were indicated as dark blue * between ouabain-perfused, stressed mice and ouabain-perfused, non-stressed mice. Black * were assigned to indicate significant differences between NaCl-perfused, stressed mice and ouabain-perfused, stressed mice as well as between NaCl-perfused, stressed mice and ouabain-perfused, non-stressed mice. Indeed, the significance levels are the same between the NaCl-perfused, stressed mice group and the ouabain-perfused, stressed as well as the ouabain-perfused, non-stressed mice. As such they were only indicated once. A detailed explanation of the significances was added to the figure legend (line 406).

Line 260, it is not clear what "c/c" means in the manuscript.

We thank the reviewer for this comment and revised this issue by replacing the abbreviation c/c by the full term "center-to-center distance" within the manuscript (line 254).

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Author(s): Lisa Rauschenberger, Susanne Knorr, Jens Volkmann, Chi Wang Ip

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