Journal of Neuroscience Methods 264 (2016) 40–46

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Short communication

Method for the assessment of neuromuscular integrity and burrowing choice in vermiform animals

C. Bainbridge, A. Schuler, A.G. Vidal-Gadea*

School of Biological Sciences, Illinois State University, 339 Science Laboratory Building, Normal, IL 61790-4120, USA

HIGHLIGHTS

- Detailed instructions for the construction of burrowing assays are provided.
- Assays permit the study of the effect of muscular exertion on locomotion.
- Assays are useful to study burrowing preference including magnetic orientation.

ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form 26 February 2016
Accepted 29 February 2016
Available online 3 March 2016

Keywords:
Burrowing
C. elegans
Locomotion

ABSTRACT

Background: The study of locomotion in vermiform animals has largely been restricted to animals crawling on agar surfaces. While this has been fruitful in the study of neuronal basis of disease and behavior, the reduced physical challenge posed by these environments has prevented these organisms from being equally successful in the study of neuromuscular diseases. Our burrowing assay allowed us to study the effects of muscular exertion on locomotion and muscle degeneration during disease (Beron et al., 2015), as well as the natural burrowing preference of diverse *Caenorhabditis elegans* strains (Vidal-Gadea et al., 2015).

New method: We describe a simple, rapid, and affordable set of assays to study the burrowing behavior of nematodes and other vermiform organisms which permits the titration of muscular exertion in test animals.

Results: We show that our burrowing assay design is versatile and can be adapted for use in widely different experimental paradigms.

Comparison with existing method(s): Previous assays for the study of neuromuscular integrity in nematodes relied on movement through facile and homogeneous environments. The ability of modulating substrate density allows our burrowing assay to be used to separate animal populations where muscular fitness or health are not visible differentiable by standard techniques.

Conclusion: The simplicity, versatility, and potential for greatly facilitating the study of previously challenging neuromuscular disorders makes this assay a valuable addition that overcomes many of the limitations inherent to traditional behavioral tests of vermiform locomotion.

1. Introduction

Burrowing is one of the most widespread forms of animal locomotion. Many organisms use burrowing as their primary form of locomotion through a wide range of substrates. For example, terrestrial vertebrates like the sandfish lizard (Maladen et al., 2009) and various species of burrowing snakes (Marin et al., 2013) use burrowing through a wide range of substrates. Burrowing is also frequently seen in subsea terrains (e.g. razor clams Winters et al., 2012). However, much progress remains to be made in order to understand this behavior. Most work to date focused on polychaetes and annelids which are behaviorally accessible but not genetically tractable (Topoliantz and Ponge, 2003; Dorgan, 2015). Unlike other forms of locomotion (e.g. swimming, crawling; Vidal-Gadea et al., 2011) burrowing takes place through environments that are often heterogeneous and therefore pose varying demands on the musculature of burrowing organisms. For example, work on burrowing on clams characterized the immense muscular effort required to burrow through heterogenous environments (Winters et al., 2012). The variable demands posed on the organism makes burrowing especially well-suited for the study of neuromuscular disorders where it can be crucial to discern the functional limits

* Corresponding author. Tel.: +1 309 438 5220; fax: +1 309 438 3722.
E-mail address: avidal@ilstu.edu (A.G. Vidal-Gadea).

http://dx.doi.org/10.1016/j.jneumeth.2016.02.023
0165-0270/

of healthy and diseased musculature. The use of burrowing assays allows experimenters to finely titrate substrate density, thus controlling the amount of muscular exertion required of test animals. Recently, we developed a burrowing assay which permitted us for the first time to conduct genetic screens that separate healthy animals from those with dystrophic muscles (Beron et al., 2015). This work restricted itself to illustrating the usefulness of this new assay without providing essential directions for its successful performance, or adaptability to additional approaches. Here we present detailed step by step directions for the successful performance of this assay to challenge and assess muscular integrity. These burrowing assays offer a reliable way to assess neuromuscular degeneration by using cost effective and easy to use materials that are equally amenable to the performance of genetic screens, as well as teaching activities. We discuss the potential for combining this approach with current force measurement approaches.

Recently, we discovered that nematodes such as *Caenorhabditis elegans* detect and orient to earth-strength magnetic fields. We adapted a T-maze assay in order to present burrowing worms with two choices and went on to show that worms use the Earth's magnetic field to guide vertical migrations in a satiation dependent manner (Vidal-Gadea et al., 2015). We describe a simple and novel set of assays designed to facilitate the study burrowing in *C. elegans* and other vermiform animals. We provide detailed instructions in the setup and execution of burrowing assays designed to test muscular integrity, study burrowing kinematics, and determine burrowing preference under differential burrowing conditions. Harnessing burrowing behavior of nematodes and other vermiform animals will provide a wealth of new insights into the study of many neuromuscular diseases as well as the understanding of animal behavior.

2. Materials and methods

2.1. Culturing and preparation of C. elegans for burrowing assays

Wild-type *C. elegans* were raised on nematode growth media (NGM) agar plates seeded with OP50 strain bacterial lawns for food as previously described (Brenner, 1974). Animals were grown at 20 °C, in a 37% humidity room, in infection-free plates and never allowed to starve or to overpopulate their plates before testing. Burrowing assays are carried out on day 1 adult animals. Animal populations were synchronized by bleaching adult hermaphrodites (Porta-de-la-Riva et al., 2012) and placing isolated eggs into agar plates. Worms have been described to rapidly accumulate random mutations (Denver et al., 2004). Behavioral parameters such as locomotion are susceptible to mutations in many genes and will show significant changes over many generations (personal observation). Therefore to prevent unwanted mutations from affecting our results we instituted a lab practice whereby every strain being worked with is replaced (from frozen stocks) every three months.

2.2. Burrowing pipette preparation

Burrowing assays are conducted in 5 mL serological or 2 mL borosilicate glass pipettes (ExactaCruz, CA) filled with chemotaxis agar (Hart, 2006). The construction of the pipettes differs depending on the intended parameter being evaluated. We have devised four different configurations which we use to study diverse phenomena: (1) Different density agars can be used to present animals with varying muscular challenges. Animals are then raced while they burrow toward an attractant in pipettes containing either different environmental conditions, or different worm strains (Fig. 1). (2) A second pipette configuration permits the study of the long term effects of varying levels of muscular exertion on muscle health and locomotion. This is accomplished by constructing pipettes filled with regular NGM (rather than chemotaxis) agar, and that have OP50 bacterial food at regular intervals to prevent starvation. (3) Assessing burrowing choice involves injecting animals into the center of pipettes filled with chemotaxis agar and equidistant from test and control stimuli at opposite ends of the pipette. (4) To film and quantify the kinematics of burrowing behavior we replace 5 mL plastic serological pipettes with 2 mL borosilicate glass Pasteur transfer pipettes which are smaller in diameter and more transparent than their plastic counterparts. This permits the clear filming of burrowing animals under different experimental conditions.

3. Results and discussion

3.1. Agar preparation for burrowing assay

The preparation of agar for burrowing studies requires some considerations unique to these studies and differing depending on the type of assay to be conducted. For short term choice assays where animals will not be tested over long periods of time (under 12 h) a standard chemotaxis agar recipe can be used (Hart, 2006) which avoids the use of salts which might otherwise interfere with the tested orientation behavior of worms. Briefly, this consists of 3% agar, 5 mM KPO_4 [pH6], 1 mM $CaCl_2$, and 1 mM $MgSO_4$ which are autoclaved for 20 min, poured into 10 cm Petri plates, and allowed to dry overnight. The amount of agar used can be modified to obtain agar of different densities. For long term assays, where worms need to survive in plates for a day or longer, we used standard nematode growth media agar (Brenner, 1974), as this agar contains all the salts and nutrients required for worms to grow and thrive. Briefly, 1 L of NGM agar is made by autoclaving of 3 g NaCl, 17 g agar, and 2.5 g peptone in 975 mL of dH_2O. After the solution cools to 55 °C 25 mL of KPO_4, 1 mL 1 M $MgSO_4$, 1 mL 1 M $CaCl_2$, and 1 mL of 5 mg/mL cholesterol (in ethanol) are added, in that order, to prevent salt precipitation at intermediate pHs. The use of NGM agar during long term assays is necessary as worms need cholesterol and salts for prolonged survival and would not develop normally in chemotaxis agar as it lacks salts and cholesterol. Because worms will be burrowing through the agar, it is imperative that it be free of salt crystals as these have the potential to harm worms as they move between them. Therefore the pH of each solution used in the agar preparation must be carefully monitored and each pipette must be inspected before and during use. It is particularly important to add the $CaCl_2$ last as calcium may fall out of solution if added prematurely. Air bubbles within the pipette must be absent as these allow worms to exit the agar and alternate between burrowing and crawling. Air bubbles can have different causes and may be introduced during the preparation of the agar. To prevent these type of air bubbles it is important to cover the glass container with the cooling agar with plastic wrap (Fig. 1A). As the agar cools in the container, the hot air within the sealed container also cools decreasing in volume following the ideal gas law ($PV = nRT$). This results in the creation of a vacuum that pulls air out of solution resulting in homogenous (bubble-free) agar. A second source of air inside agar pipettes is time. Agar pipettes may be stored in a 4 °C refrigerator for short periods of time but must be used within one or two weeks from their construction (ideally no later than two or three days). Eventually, agar within the pipettes will dry and shrink, creating air pockets over which worms are able to crawl freely. Similarly, an additional source of air pockets are the holes bored into the pipettes to accept chemicals or animals. These holes need to be made as close to the start of the assay as possible to prevent agar from drying and introducing air pockets in the pipettes.