Rodent and fly models in behavioral neuroscience: An evaluation of methodological advances, comparative research, and future perspectives

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ABSTRACT

The assessment of behavioral outcomes is a central component of neuroscientific research, which has required continuous technological innovations to produce more detailed and reliable findings. In this article, we provide an in-depth review on the progress and future implications for three model organisms (mouse, rat, and Drosophila) essential to our current understanding of behavior. By compiling a comprehensive catalog of popular assays, we are able to compare the diversity of tasks and usage of these animal models in behavioral research. This compilation also allows for the evaluation of existing state-of-the-art methods and experimental applications, including optogenetics, machine learning, and high-throughput behavioral assays. We go on to discuss novel apparatuses and inter-species analyses for centrophobism, feeding behavior, aggression and mating paradigms, with the goal of providing a unique view on comparative behavioral research. The challenges and recent advances are evaluated in terms of their translational value, ethical procedures, and trustworthiness for behavioral research.

1. Introduction

Understanding aspects of behavior that can be ascribed to the function of specific genes or brain circuits is one of the most important underpinnings of neuroscience research (Sokolowski, 2003; Sousa et al., 2006). However, identifying factors that might influence a complex behavioral trait, such as anxiety or depression, is not simple. It requires navigating through numerous behavioral assays and animal models to uncover, piece by piece, the underlying characteristics of different neuropsychiatric disorders. Thus, reviews of the animal behavior literature are particularly important, as they provide up-to-date discussions on protocols and methodological recommendations (Brown and Bolivar, 2018; Sousa et al., 2006), evaluations of particular disease models (Janus and Welzl, 2010; Silverman et al., 2010), comparisons of strain differences (Deacon et al., 2007; Einat, 2007), and guidance on specific experimental paradigms (Brooks and Dunnett, 2009; Osaki et al., 2015).

Nevertheless, few reviews consider a broad spectrum of behavioral assays, and even fewer discuss their implications across animal models. With this in mind, we aim to provide a cross-species overview of predominant behavioral assays, technological innovations, and future perspectives. The first section discusses why rodents and flies are the most employed non-primate animal models for behavioral research. By manually screening highly cited methodological articles or reviews, we compiled comprehensive lists of popular behavioral assays for these models, encompassing more than 150 tasks for rodents and flies. Then, we evaluate modern methods and apparatuses, which are pushing the boundaries of behavioral neuroscience, such as optogenetics, machine learning, and high-throughput behavioral assays. Next, we examine novel technologies for centrophobism and feeding behavior that allow for the assessment of inter-species behavioral commonalities. The final sections deliberate on the future of the field and consider emerging themes, including the translational value of neuropsychiatric models.
and Memory; Social Behavior; and Exploratory and Emotional Behavior. This categorization was maintained for the rodent (Supp. Table 1) and fly (Supp. Table 2) behavioral lists. The compiled behavioral assays focus on those for fully developed animals, thus only tasks performed using adult Drosophila were considered. By analyzing the assay list for each model (Fig. 2), we can observe that, despite the existence of a greater number of rodent tasks, the proportion of Drosophila assays created in each category is the same (chi-squared test, $p = 0.986$). This result indicates that, although there is a preference for the development of rodent behavioral methods, there is no apparent limitation for the creation of Drosophila assays in any given field of behavioral research.

3. State of the art in behavioral research

Historically, the behavioral neuroscience field has benefited from many biotechnological advances, some of which were paired with behavioral tests to measure physiological states and uncover changes in the nervous system underlying behavioral paradigms (e.g. brain oscillations, neuronal firing, neurotransmitter balance, etc.). As an example, in vivo electrophysiology methods laid the foundation for understanding the connection between brain activity and animal behavior. Over the years, the number of important findings, together with constant equipment improvements, gradually made these methods central for numerous laboratories (Svensson et al., 2018; Taketani and Baudry, 2006). Additionally, new technologies, like the imaging of neuronal activity through ion-dependent fluorescent indicators, are pushing the boundaries on the study of circuits responsible for behavior, by evaluating neuronal function at both the cellular and population levels (Kerr and Denk, 2008). Some emerging methods and apparatuses provide novel ways to measure specific outcomes, improve robustness in assessments, and even bring new insights on ethological paradigms of a given species. With this in mind, we discuss technologies changing the status quo on behavioral research.

3.1. Optogenetics: integration of behavioral assays with neural control

Behavioral functions emerge from neuronal networks that produce movements and organize them into the appropriate temporal and spatial patterns to generate a given action. Few techniques have contributed as much to our understanding of this process as optogenetics, which is based on the activation of light-sensitive ion channels or proteins, genetically engineered for targeted-cell expression. This method allowed researchers to examine the consequences of the excitation or inhibition of distinct circuitry on physiology and behavior. It became a highly-used approach for a variety of animal models, including rodents (Deisseroth, 2015) and flies (Kazama, 2015). Notably, alternative methods to optogenetics are still successfully employed to silence or activate neurons in a spatial and temporal manner. For Drosophila models, a powerful approach is the use genetically encoded channels that permit neuronal activity modulation in response to temperature changes, also called thermogenetic tools. For example, the expression of the temperature-sensitive variant GAL80 in flies allows for the temporal control of GAL4-driven gene expression, which when combined with adequate UAS-promoted genes can modulate neuronal activity. Many options are currently available to achieve this effect, such as the inhibitory potassium channels Kir2.1 (Baines et al., 2001), EKO (White et al., 2001), DORK (Nitabach et al., 2002), or the excitatory temperature-sensitive transient receptor potential (TRP) channels, like the heat-activated dTrpA1 (Hamada et al., 2008), or the cold-activated TRPM8 (McKemy et al., 2002). Nevertheless, this technique has inherent challenges that should be considered. First, the temporal resolution of neuronal control is usually limited to a scale of minutes to hours (Bath et al., 2014), and more importantly, the changes in environmental temperature can interfere with behavioral outcomes (Ki et al., 2015). Lastly, the use and development of thermogenetic tools remain largely unexplored in other organisms, as keeping the adequate
temperature range is particularly complex in mammals (Bernstein et al., 2012).

Unlike pharmacological, thermogenetic, electrical or lesion-based interventions, optogenetics mutually provides cell-specific and millisecond-resolution modulation of the activity of defined neuronal populations, opening a new horizon of possibilities for brain circuitry control when performing behavioral studies. For instance, optogenetic targeting has numerous advantages over electrical stimulation, which is widely used for rodents models. It is cell-type specific, has greater temporal and spatial precision (Packer et al., 2013), and allows bidirectional control of membrane potential and firing (Newman et al., 2015). An example demonstrating the advantages of site-specific optogenetic targeting compared to nonspecific electrical stimulation comes from the study of aggression regulation from the ventrolateral portion of the ventromedial hypothalamus in mice. Optogenetic, but not electrical, stimulation of the ventrolateral portion of the ventromedial hypothalamus in mice elicited the attack behavior on cage intruders (Lin et al., 2012). In that study, no specific optogenetic cell targeting was used, and the placement of both electrical electrodes and viral vectors were guided through stereotaxic surgery. However, only the optogenetic stimulations could provoke the attack phenotype, while electrical stimulations resulted in freezing and flight behavior resulting from the stimulation of adjacent nuclei.

Modern apparatuses, which include closed-loop feedback based on experimental conditions (Grosenick et al., 2015), use optogenetics not only as an auxiliary methodology, but as a gear of the behavioral paradigm. By coupling computational control to detection systems, it is possible to deliver light stimulation instantly after behavioral changes and actions, developing novel operand-like tasks associated with neuronal control. An example of this approach is the use of optogenetic self-stimulation of dopaminergic neurons of the ventral tegmental area to mimic the action of addictive drugs, where all experimental mice acquired the self-stimulation behavior (Pascoli et al., 2015). Additionally, a recent study used position tracking to automatically activate or inhibit OLMz2 neurons from mice hippocampus in the object recognition assay, stimulating these cells specifically during exploration of one of the two objects presented, ultimately demonstrating that these neurons could bi-directionally modulate object memory learning (Siwani et al., 2018). Previously, a similar principle of position tracking was developed to optogenetically stimulate multiple freely-moving Drosophila, where the authors performed an operand assay in which a courting male fly learned and formed a long-lasting memory to avoid a virgin female, by conditioning the approach with acute punishment (Wu et al., 2014). Optogenetic feedback systems are not limited to operand tasks, they can also provide simulated endogenous physiological states for different types of behavioral paradigms. For instance, a series of studies in mice demonstrated that optogenetic activation of the neural representation of a context can substitute different types of experience and fabricate memories in a similar way to natural exposure to stimuli (Okuyama et al., 2016; Pignatelli et al., 2019; Ramirez et al., 2015, 2013). In fruit flies, closed-loop optogenetic activation and silencing was recently used to induce appetitive or aversive feeding preferences by modulating gustatory neurons independent of the eaten food, effectively generating virtual taste perception in flies (Moreira et al., 2019). In these examples, different from simply disrupting or activating neuronal pathways that process behavior, the optogenetic stimuli evoked specific sensory representations of the animals’ past experience, ultimately generating a new type of behavioral experience. Beyond excitation and inhibition of ionic channels, the use of light-activated biochemical signaling (Airan et al., 2009) is gradually increasing. As an example, the creation and testing of Opto-αAR, which is obtained by replacing the intracellular loops of bovine rhodopsin with those of the mammalian adrenergic receptors (Kim et al., 2005), revealed that optical activation of α1 adrenergic receptor pathways in NAc neurons is appetitive by evoked place preference (Airan et al., 2009), whereas optical activation of β2 adrenergic receptor pathways elicits anxiety-related behaviors (Siuda et al., 2015). Undoubtedly, the development and application of these light-activated receptors will bring important tools for the study of metabotropic neurotransmitter systems.

However, optogenetic tools are not free from caveats. For freely-moving behavioral experiments, mice and rats should have an intracranial implants so the light-sensitive channels can be activated (Cardin et al., 2010; Stirman et al., 2012). This invasive procedure, as the subsequent tissue damage and surgery recovery, can induce behavioral changes. The light delivery is commonly done through a fiber-optic patch cord, which can constrain the animal’s locomotion due to limited reach or accumulated torque in the fiber (Klorig and Godwin, 2014). Moreover, it has been shown light stimulation of the brain generates heat, which changes neuronal activity patterns and cellular viability (Owen et al., 2019; Peixoto et al., 2020). For Drosophila, the use of high-power laser/ LED stimulation is necessary, as the optogenetic stimulation for behavioral experiments is typically done through the cuticle, which can also cause cellular damage. Moreover, to prevent the unspecific activation of some highly light-sensitive channels by normal light/dark housing conditions, flies are often reared in total darkness or under blue-light light/dark cycles (as red-shifted wavelengths are commonly used for stimulation). It can bring a range of behavioral confounders, as blue light was shown to be aversive to flies (Lazopulo et al., 2019), and long-term blue-light exposure was reported to reduce survival and causing neurodegeneration in this organism (Nash et al., 2019). Nevertheless, with all pitfalls considered, optogenetic tools are unquestionably among of the most powerful existing techniques for the investigation of neuronal circuits governing behavior.

3.2. Machine learning

Recent advances in computational power are allowing for the collection of large data sets of behavioral measurements. Currently, many video-analysis systems are capable of tracking single or multiple animals over a given task, providing continuous information about the position and locomotion of each animal (Colomb et al., 2012; Gomez-Marin et al., 2012; Patel et al., 2014; Scaplen et al., 2019). However, the assessment of such screenings has been mostly limited to tracking-based quantifications, as the analysis of the numerous parameters in complex behaviors poses a challenge to the existing algorithms. An emerging tool to handle the interpretation of such intricate data is machine learning, which is gradually being applied in both laboratory and wild animal research (Egnor and Branson, 2016; Valletta et al., 2017).

Most current methods are based on supervised machine learning, which relies on training steps using manually-labeled inputs to identify data patterns according to the given classification. It is used in many fields of biomedical behavioral research, including social interaction (Robie et al., 2017), freezing behavior (Amorim et al., 2019), locomotion- and position-based complex behaviors (Geuther et al., 2019; Kabra et al., 2013), grooming behavior (Qiao et al., 2018; van den Boom et al., 2017), and vocal communication (Burkett et al., 2015). Moreover, recent programming toolboxes are helping to expand these applications by facilitating the development of new analyses based on this type of algorithm (Arac et al., 2019). However, the quality of the classification is highly dependent on the provided dataset, as these methods can systematically incorporate pre-existing subjective criteria. This can be especially problematic in fields where the use of different methodologies leads to disagreements on appropriate behavioral classifications (as discussed in Marques et al., 2016).

A growing alternative is the use of classification methods incorporating unsupervised learning algorithms to objectively discover behavioral structures in non-labeled datasets (Berman et al., 2014; Klibaité et al., 2017; Marques et al., 2018; Wiltschko et al., 2015). An example of fruitful usage of such approach is the improvement of classical tracking systems, by accurately determining the trajectories of unmarked individuals within large groups of animals (Pérez-Escudero et al., 2014;
Beyond the enhancements in animal identification, unsupervised machine learning has been successful in mapping of novel behavioral patterns that are not observable by human scoring, based on video recordings of mice and flies (Berman et al., 2014; Wiltshko et al., 2015). Moreover, it can identify features that predict particular behavioral actions, as a recent study in fruit flies reported that the structure of walking behavior can be decomposed into a small number of patterns that can be used to reveal probable trajectories in locomotion (Katsov et al., 2017). In combination with optogenetic techniques, unsupervised learning was incorporated to uncover connections between neuronal activation and behavior in Drosophila larvae (Vogelstein et al., 2014). However, unsupervised machine learning methods also have implementation setbacks. The boundaries of the behavioral sorting are not rigidly defined, which can cause multiple valid outputs for the pattern separation, even on the same dataset (Valletta et al., 2017). Depending on the input, it is possible that the categorization will miss context-specific behavioral classes, or that steps of a multipart behavior would split into different groups. Lastly, some algorithms require threshold settings to define the number of classes, bringing subjectivity to the classification process. Thus, although unsupervised methods provide a way to discover behavioral patterns without the need for prior sorting, the output can have vast differences and uncertain biological value, depending on the computational approach.

It is clear that behavioral applications of machine learning technology will likely become a common tool in neuroscience research. Software toolboxes and interfaces based on machine learning already pose a realistic option for many laboratories, with user-friendly systems and interpretable results (Arac et al., 2019). Additionally, the discovery of subtle behavioral differences, previously undetectable with traditional methods, will surely bring new discussions and insights to animal behavior research.

### 3.3. High-throughput behavioral assays

Automated analyses increase not only the number of measured variables in behavioral tasks, but also the data precision, reliability, and most importantly, experimental throughput. Until recently, the techniques necessary for large-scale assays of animal behavior were unavailable, and most of the assessments were—and often continue to be—manually scored. For the Drosophila model, paradigms as such as grooming, food preference, and different aspects of courtship were largely neglected, as behavioral studies traditionally rely on observations made by a human observer, which is laborious and time-consuming. This clearly contrasts with the benefits of Drosophila as a high-throughput experimental model. Rodent models procedures were not much better in optimizing experimental outputs, and perhaps remain even more dependent on the manual assessment of trained researchers. Standard rodent protocols typically focus on uncovering minutiae of particular behaviors, rather than increasing throughput, thus missing many behavioral dimensions that could be simultaneously assessed.

Due to the aforementioned computational advances, this scenario seems to be rapidly changing and various high-throughput behavioral systems are under development. Video-based software methodologies were particularly innovative in this sense, as several existing methods can score behaviors from dozens to hundreds of flies simultaneously, for both singly-housed and groups of animals (Scaplen et al., 2019). Other data recording alternatives are also being tested, like a shuttle habituation system integrated with sound-based scoring that was recently used for the assessment of nearly 300 genes implicated in intellectual disability (Fenckova et al., 2019). Additionally, rising methods for quantifying behavior combining high-throughput video tracking of individuals with machine learning will provide ways to increase efficiency not only for the assay, but also for the behavioral scoring (Klibaite et al., 2017). Also, advanced robotics allowed for the development of an automated platform for handling and phenotyping, which made possible the mechanized collection of virgin females and execution of complex social behavior experiments (Alisch et al., 2018). Although the accessibility of some of these innovative equipment and software technologies are limited by the technical abilities required for their implementation, they are the first step for future user-friendly, completely automated, large-scale fly behavioral systems.

In rodent research, computerized behavioral apparatuses are starting to replace time-consuming and labor-intensive human implementation of complex assays, such as tasks of learning and memory (Erskine et al., 2019; Fredriksson et al., 2019; Poddar et al., 2013; Zou and Li, 2019). Additionally, home-cage monitoring systems are supporting the exploitation of new complex paradigms and improving the assessment of behavioral outcomes, completely integrated with high-throughput phenotyping pipelines. For instance, a promising approach has been video tracking paired with radio-frequency identification (RFID) of individual animals. RFID tags can identify different mice in naturalistic housing environments, allowing for animal-specific automation of behavioral tasks. Moreover, these tags are injected sub-cutaneously and usually do not require surgery, reducing animal distress. The method has been successively employed in several high-throughput tasks, such as social analysis (Pelch et al., 2019; Weisbrad et al., 2013), operant conditioning (Eerkes et al., 2019), olfactory phenotyping (Reinert et al., 2019), and motivation-based behaviors (Boluños et al., 2017; Santos et al., 2006).

By recording home-cage data, it is possible to avoid anxiety-related biases caused by handling or the test environment. It also allows for longitudinal locomotion and activity studies (Bains et al., 2018), able to analyze complex disease-related behaviors (Steele et al., 2007; Zarringhalam et al., 2012), and even rodent social interactions (Bains et al., 2016; Hong et al., 2015). However, as with pioneering methods for Drosophila, new specialized equipment for rodent behavioral analysis are costly, requiring substantial infrastructure and skills for their proper application. Likewise, the expansion of machine-learning technologies is necessary for handling the large multidimensional datasets generated (Hillar et al., 2018; Valletta et al., 2017). Yet, if integrated, such technologies can deliver unbiased, longitudinal, and complex data analysis for both known and novel behavioral phenotypes.

Large multidimensional and high-throughput behavioral procedures bring new and challenging issues in terms of phenotype analysis. First, these automated analyses allow for the exploration of numerous hypotheses and variables, requiring greater rigor in experimental design and statistical approaches. Second, many of these apparatuses are self-manufactured or personalized; thus a more detailed description of all the experimental parameters and procedures must be incorporated in the studies to ensure standardization and reproducibility. Moreover, it is necessary to document the animal handling, randomization, and blinding arrangements. Features that have been largely underreported in behavioral studies (Carreiro et al., 2018; Macleod et al., 2015), and that are vital to the transparency of high-throughput tasks. Finally, increasing the complexity of the assessed behaviors can bring several pitfalls to data interpretation and modeling. Behavior is an intricate combination of spatial and temporal actions from an organism facing specific inputs, so current protocols unsurprisingly focus on measuring simple behavioral patterns. As we seek to understand how multiple components of behavior are tied together, novel ethological models that can represent this complexity must be developed.

### 4. Identification of cross-species parallels by new technologies

Novel behavioral assays are allowing for the analysis and characterization of cross-species parallels, generating valuable findings for evolutionary, comparative, and translational studies. Such tools assess behaviors that are conserved across animal models and sometimes even relatable to human conditions. The following sections describe examples of how new technologies are contributing to the understanding of...
similar behaviors between fly and rodent models.

4.1. Feeding behavior

Food intake and feeding behavior are central to the survival of all animals. It is closely linked to multiple physiological processes such as reproduction, longevity, foraging, metabolism, aggression, sleep, and circadian rhythms. However, measuring food intake in *Drosophila* has been notoriously hard due to their small size and the minuscule amount of food flies consume. Until recently, the methods for monitoring food intake were limited to time-consuming laborious manual methods that mostly reported bulk food intake in groups of flies (Itskov and Ribeiro, 2013; Murphy et al., 2017; Williams et al., 2014, 2016, 2020). In addition, these methods failed to resolve the temporal dynamics of food intake as well as the fine temporal structure of feeding behavior in flies.

The situation changed when new experimental approaches were developed using a number of novel technologies, such as capacitive touch sensors to detect the physical interaction between a fly and the food (FlyPAD, Itskov et al., 2014); voltage drop assessments of the fly during feeding (FLIC, Ro et al., 2014); video-based tracking of the CAFE assay (ARC, Murphy et al., 2017); and linear CCD sensors for individual meal-bouts (Expresso, Yapici et al., 2016).

Our group was part of the flyPAD (fly proboscis and activity detector) development, which was based on the idea of creating a one-dimensional tracking system that gives a high throughput unbiased measure of the fly’s feeding behavior. Such a method could overcome the difficulties of the approaches based on video tracking, namely inefficient data analysis, low throughput and large space requirement for measuring the behavior of multiple flies simultaneously. We discovered that the signal was very data-rich and had striking rhythmicity, which we later confirmed to be individual proboscis extension/ retraction cycles during feeding (Itskov et al., 2014). These signal units were termed sips (Fig. 3A). This behavior appeared mostly when flies were feeding on solid food (1% agarose) and was much less prevalent and even unreliable when liquid food was presented.

The frequency and the shape of the distribution of the inter-sip-intervals looks extremely similar to the inter-lick-intervals histogram in mice and rats (Figs. 3B and C). This observation prompted us to look deeper into the potential similarities between the licking behavior in rodents and sipping in *Drosophila*. We have adopted the metrics and developed a characterization of licking microstructure and implemented them in the data analysis pipeline (Itskov et al., 2014). Similar to rodents, the sips in flies tend to be grouped into bursts. The bursts, in turn, can be grouped together into epochs of uninterrupted interaction with the food source, which we called activity bouts – known as clusters in rodent literature (Fig. 3C). The duration of the bursts and the distances between the activity bouts appeared to be differentially affected by the fly’s internal state. Mild and strong starvation differentially affected the duration of the feeding bursts and the distances between the activity bout IBI (inter-bout-intervals) (Fig. 3D). This result suggested the presence of distinct neuronal populations controlling various aspects of feeding behavior, such as feeding burst duration and activity bout.

There is a vast literature on the effects of various drug families on licking and food intake in vertebrates. The striking similarity of licking in rodents and sipping in flies opens possibilities for comparative studies, while the availability of the metrics and automated tools to monitor feeding behavior in flies significantly lowers the entry barrier into this emerging field. Future experiments in *Drosophila* examining the effects of various pharmacological effectors on feeding microstructure will demonstrate to what extent the mechanisms underlying the control of food intake are conserved across invertebrates and vertebrates.

4.2. Open field centrophobism

The open field test is a simple task developed to measure locomotor and emotional changes in rodents (Walsh and Cummins, 1976). Shifts in activity and the tendency to avoid the center in open field tests are used as scores to evaluate anxiety-like reactions and exploratory behavior in rodents (Seibenhener and Wooten, 2015). Animals with centrophobism, i.e. spending less time in the central part of the field, are regarded as more fearful and anxious. Interestingly, fruit flies show a behavioral pattern similar to that of mice and rats in the open field. The centrophobism behavior in *Drosophila* was first reported more than 30 years ago; however, it was in a specific context (Gott and Biesinger, 1985a, 1985b). Briefly, flies previously stressed by diethylether exposure avoid the center of a round arena, despite the placement of olfactory, thermal, or visual attractants in the maze center. These results were obtained using an analog object finder, in an unique set-up specially designed for...
individual fly tracking experiments ( Bölthoff et al., 1982 ). It required the much later development of accessible digital tracking systems for the verification of spontaneous centrophobism in Drosophila, in a study that observed an innate propensity of the flies to walk near the periphery of a vertically-limited square arena (Fig. 4A; Martin, 2004). Another leap in the technological advance for freely-moving fly tracking was the tridimensional recording of the fly position by multiple-camera setups. It can be combined with different tracking methods, such as fluorescent reporters (Ardekani et al., 2012), 3D-holographic microscopy techniques (Kumar et al., 2016), or intricate algorithms for sequential processing of numerous frames (Ardekani et al., 2013). For instance, a study using green fluorescent protein (GFP) expression as a reporter to aid the tracking of a single fly (Ardekani et al., 2012) demonstrated that the wall-proximity behavior is maintained even when the Drosophila is allowed to fly unconstrained inside a vial (Fig. 4B).

The similarity between model organisms goes even further: temporal analysis of the locomotion showed that when placed in the open field arena, flies and rodents have an initial period of elevated activity, followed by a decrease in activity to a baseline level (Liu et al., 2007). Moreover, it has been demonstrated that the neurobiology of this behavior is common across these species. By using comparable genetic and pharmacological manipulations that can interfere with centrophobism, and correlating the observed effect sizes from such treatments, it was observed that the Drosophila behavior shares underlying neurogenetic pathways with mammalian anxiety (Mohammad et al., 2016). Such inter-species studies contribute to the translational understating of anxiety-related disorders and may describe evolutionarily-conserved molecular pathways that might be explored in future treatment research.

4.3. Aggression and mating interplay

Aggression is a complex goal-directed behavior present in virtually all social species. It is crucial for animal self-preservation, maintenance of resources and, ultimately, survival. Decades of research have identified several neurotransmitters, neuropeptides, and brain circuitries responsible for this behavior in many animal models (Comai et al., 2012; Nelson and Trainor, 2007). However, the degree to which such mechanisms are conserved across species remained largely unexplored until recently (Thomas et al., 2015). Additionally, mating behavior has been shown to be closely linked to aggression in a number of animal models, as the decision whether to mate or fight is dependent of the same brain pathways, but driven by different internal states of the organism (Anderson, 2016). Novel assays combining behavioral tests and optogenetics, together with the development of high-throughput tasks, opened a new door to explore the regulation of aggression and mating, indicating a surprising level of similarities among rodent and Drosophila models.

Classical experiments in rodents pointed to the existence of specific areas in the medial hypothalamus, which when electrically stimulated
were able to trigger attack behavior (Kruk, 2014). Recently, by the use of anatomically precise viral-vector injections or steroid hormone receptors as promoters for the expression of optogenetic channels, the neurons responsible for this activity in the ventromedial hypothalamus (VMH) could be identified (Lee et al., 2014; Lin et al., 2011). Specifically, it was shown that optogenetic, but not electrical, stimulation of ventrolateral subdivision of the VMH causes male mice to attack both females and inanimate objects, as well as males (Lin et al., 2011). Further optogenetic manipulations identified the oestrogen receptor 1-positive (ESR1+) neurons of this area to be sufficient to initiate attack and continuously required to be active during aggressive behavior. Interestingly, weaker optogenetic activation of these neurons promoted mounting behavior towards both males and females, indicating that the same circuit can regulate different outcomes of a social encounter (Lee et al., 2014).

For Drosophila models, research on the circuitry that specifically controls aggression were long limited to small-scale screenings of specific neurotransmitter-expressing neurons. Nevertheless, some important neuromodulators of this behavior were identified, such as octopamine (Zhou et al., 2008), dopamine (Alekseyenko et al., 2013), serotonin (Alekseyenko et al., 2014), Neuropeptide F (Dierick and Greenspan, 2007), and the Drosophila Tachykinin (TK) (Asahina et al., 2014). Interestingly, TK homologues (including the mammalian Substance P) have also been pointed as regulators of aggression in several rodent models (Felipe et al., 1998; Halasz et al., 2009; Shaikh et al., 1993), and Tachykinin receptor 1 is expressed in the mouse VMH ESR1+ neurons, shown by optogenetic-behavioral assays to specifically control mounting and aggression (Lee et al., 2014).

The evidence of commonalities between species was further strengthened by a seminal study performing high-throughput behavioral assays computed by machine-learning algorithms (Hoopfer et al., 2015). Using the thermosensitive ion channel dTrpA1, over 3000 GAL4 lines were screened to identify circuits that promote aggression, a subpopulation of 8–10 P1 neurons per hemibrain. Notably, as the rodent VMH ESR1+ neurons (Lee et al., 2014), activation of P1 Drosophila cells was able to incite both increased aggression and male-male courtship behavior.

Taken together, these results indicate a remarkably similar regulation and circuitry organization between rodents and flies, as both models display neuronal populations which receive inputs from homologous neuropeptide pathways and promote internal states that modulate the engagement in either sexual or aggressive behaviors (Fig. 5). Future research is necessary to show if the parallels between the ESR1+ VMH and the P1 clusters represent conserved neuronal populations (Anderson, 2016).

5. Challenges and future perspectives

5.1. The translational value of cross-species behavioral research

Translational studies from neuroscience typically investigate the effectiveness of new strategies for prevention and treatment of brain disorders (Ehrenreich, 2004). However, recent analyses of the translational value for disorders like stroke, multiple sclerosis and
amotrophic lateral sclerosis show that the number of pre-clinical pos-
itive results that were applicable to humans is negligible (O’Collins et al., 2006; Scott et al., 2008; Vesterinen et al., 2010). Reproducing important findings not only across strains, but also across species, is a way to increase the robustness of these discoveries, and may be one of the solutions to improve the success – as well as uncover the limitations – of basic research contributions.

Research on Fragile X syndrome (FXS) is a fruitful example of posi-
tive translational outcomes by studying behavioral disorders across species, using rodents and flies. It started with the description of a highly-conserved homolog to the mammalian FMRI gene in Drosophila. By mutating this gene, it was observed that flies have similar behavioral impairments as FXS humans or mice. For instance, they are unable to maintain a normal circadian rhythm in constant darkness and exhibit irregular patterns of locomotor activity (Dockendorff et al., 2002). Resemblances between human and fly FXS effects opened the door to study potential routes for pharmacological intervention. Fly studies showed that feeding metabotropic glutamate receptor (mGluR) antagonists to FXS mutants rescued defects in behavior and brain circuitry (McBride et al., 2005). This phenotype rescue was later reproduced in rodent models by reducing mGluR5 activity (Bhogal and Jongens, 2010). By the use of these models, other pharmacological targets were also identified. This lead to clinical trials, some still ongoing, investigating the use of glutamate receptors antagonists – especially of mGluR5 – and GABAergic agonists to treat this condition in humans (Erickson et al., 2017). Ultimately, even if the clinical trials do not demonstrate the ef-
cicacy of this pharmacological treatment, the rigorous translational approach will provide unique and valuable lessons on the similarities and limitations of these neuro-behavioral disorder models.

Additionally, novel apparatuses can better characterize similar behavioral components across species, which are essential for medical translation from animal models to humans. For example, great progress has been made by touchscreen-based cognitive tasks for rodents (Nithianantharajah and Grant, 2013). In this method, a display sensitive to touch is presented to a trained animal, which makes choices by nose-poking specific objects on the screen, a process that is comparable to those employed for human touchscreen batteries (Bussey et al., 2012). By measuring with similar protocols, cognitive components of rodents and humans can be directly matched and analyzed. For instance, be-
haviors as visual discrimination, spatial learning, and visual attention have been described to show comparable effects for the same mutations across these species (Nithianantharajah et al., 2013). To this point, most of the translational human therapies for cognitive disorders were tested based on rodent-specific tasks, that may not relate to the same behav-
ioral components to the ones assessed in humans (Bussey et al., 2012). The development of more relatable methods, such as the touchscreen approach can provide new insights to the neurobiology of cognition.

The combination of genetic models and comparable tasks for trans-
slational research of behavior is promising, but limitations should be kept in mind. First, similar mutations in homolog genes do not necessarily mean that the proteins of given organisms undergo the same functional changes, so additional molecular analyses are recommended to validate these models. Second, a broad standardization of the behavioral proto-
colos is necessary to allow for the adequate interpretation of findings from different reports. Nonetheless, future studies in the field can bring priceless knowledge for the pursuit of genetic correlates of behavior across species and, in combination with high-throughput or machine learning techniques, optimize the search for novel target genes and proteins for the treatment of behavioral disorders.

5.2. Replacement, reduction and refinement

The three ‘R’s are principles for guiding the ethical use of animals in scientific research. Replacement refers to the development of methods that avoid or replace the use of animals; Reduction is the optimization processes aiming to minimize the number of animals used per experiment; and Refinement discusses protocols that minimize animal suffering and improve welfare. They were first described in 1959 (Russell et al., 1959), and the concept laid the foundation of several modern legislations on laboratory animal handling and care (Combes and Balls, 2014). Moreover, there is an increasing interest for the fast adoption of these concepts in animal research (Carlsson et al., 2004) and a continuous discussion on strategies for improving animal welfare (Prescott and Lidster, 2017).

The behavioral research field is highly dependent on laboratory animals, and such studies have been primarily based on rodents (Fig. 1). By the very nature of some behavioral tasks, the experimental proced-
ures are likely to inflict harm or discomfort on the studied animals (Vieira de Castro and Olsson, 2015). Nevertheless, these approaches are still necessary, as behavioral research is critical for facing the current challenges in human health. Mapping genetic, metabolic and physi-
ological components of complex behavioral features can lead to new treatments for increasingly prevalent disorders like depression, addiction, anxiety-based impairments, or neurodegenerative diseases. Thus, finding a balance between growing ethical concerns and neuroscientific advances is a timely matter.

A viable alternative to rodent models has been the use of in-
vertebrates, such as the fruit fly, Drosophila melanogaster. The fly has already been a useful organism for studying several biological processes including genetics, development, molecular and cell biology, immu-
nology and neuroscience. Despite the ancient evolutionary separation of arthropods from vertebrates (Adoutte et al., 2000), the genetic and molecular components are remarkably similar (Rubin et al., 2000). Thus, while flies and humans have obvious anatomical differences, they have striking parallels on the level of biological processes. For example, several neurobiological processes are comparable, including membrane excitability, neuronal signaling and shared classes of neurotransmitters (O’Kane, 2011), likely because of common evolutionary origin of the central nervous system from flies and humans (Hirth and Reichert, 1999). Moreover, there is considerable genetic overlap, as close to 75 % of all human disease genes have relevant homologs in the Drosophila genome (Rubin et al., 2000). Accordingly, we could also observe that, during the last years, a number of behavioral methods are being developed for flies within all subfields of behavior assessment (Fig. 2).

To the best of our knowledge, the invertebrate models do not process distress in a similar manner as vertebrates, while having the biological machinery to instinctively respond to environmental stimuli. This means that organisms like the fly may be ideal for studying the molecular basis of evolutionarily conserved behaviors. The development of behavioral assays capable of validating the phenotypic similarities across species, together with the growing vertebrate ethical awareness, make the use of such models very attractive, mainly on high-throughput, animal-inten-
sive, behavioral investigations. For instance, large genetic or pharma-
cological screenings can serve as a stepping stone for finding target pathways that can be then further explored in rodents models (Fencova et al., 2019; Pandey and Nichols, 2011). Thus, we believe that the growing compliance to ethical principles as the three ‘R’s will drive the expansion of the use of flies to study behavioral paradigms in the next years. With the progression of automated assays, it is a reasonable possibility that invertebrates could become the first-choice model for large genetic and pharmacological screenings.

5.3. Reproducibility and robustness of behavioral experiments

The low reproducibility rate of scientific articles has been under intense debate in the scientific community. A recent questionnaire, which surveyed more than 1500 researchers, showed that over 70 % of the responders have tried and failed to reproduce another scientist’s experiments, and more than 50 % were unsuccessful in reproduction of their own experiments (Baker, 2016). Additionally, rodent behavioral phenotyping has arguably been one of the most controversial fields in this discussion (Bespalov and Steckler, 2018; Fonio et al., 2012).
Systematic analyses in several neurobehavioral fields such as stroke, multiple sclerosis and amyotrophic lateral sclerosis have been indicating that preclinical models are not robust enough to translate their findings into clinical efficacy (O’Collins et al., 2006; Scott et al., 2008; Vesterinen et al., 2010). Many experimental issues that can contribute to this problem have been pointed to as common in behavioral research, such as the lack of proper training and poor study design (Bespalov and Steckler, 2018), underpowered and underreported results (Carneiro et al., 2018; Macleod et al., 2015; Moulin et al., 2020), experimenter biases (Chapman et al., 2018; Holman et al., 2015), and poor characterization of the animal models (Perrin, 2014).

There are no simple solutions for these issues, but some of the technological advances mentioned in this review can undoubtedly provide new ways to improve the robustness and reproducibility of behavioral experiments. First, the widespread use of automated systems can reduce the variability caused by subjectivity assessments, experimenter biases or non-standardized procedures. Moreover, the increase in the range and sensitivity of measured parameters can help to expand the current testing of simple paradigms that mostly fail to exemplify the complexity of human behavior. Some automated apparatuses can even reduce the number of external confounders and make protocols more straightforward, such as the rodent home-cage monitoring technologies that avoid the customary requirement of training and acclimating animal for multiple days. Additionally, the use of available online ‘big data’ methods has been suggested as a tool for implementing widespread animal study registries – as currently required for clinical trials (Baker, 2019), and for constructing large phenotyping databases for developers and users of behavioral models (Fonio et al., 2012).

We should note that the reproducibility debate has yet to grow outside rodent-based research. Comparative analyses of reliability between Drosophila behavioral methods – such as done by Deshpande et al. for food intake (Deshpande et al., 2014) – are rare. Additionally, the trustworthiness of analytical methods are usually evaluated on an ad hoc basis, such as for genomic analysis (Sugden et al., 2013), but overall debates on the matter are still limited. Even though the emerging behavioral technologies will likely improve behavioral protocols in fly research as well, future validations are crucial for increasing the robustness, confidence, and ultimately usage of this animal model.

We can observe a substantial increase in neuroscience publications developing and using open-source tools. This movement can become an important support for reproducibility improvement of behavioral experiments, as previously largely inaccessible state-of-the-art methods can now be implemented in a cost-effective manner in a broad number of laboratories (White et al., 2019). Moreover, online platforms, such as the Open Behavior Project (openbehavior.com), are being created for dissemination open-source projects in behavioral research, containing dozens of resources, like hardware blueprints and freely-available software. These efforts are certainly promising, but additional platforms and incentives are still needed to stimulate sharing the development, standardization, and replication of open behavioral assays.

6. Final remarks

In this study, we reviewed the current state of the behavioral assays for rodents and flies, discussed some of the cutting-edge techniques of the field and, in light of these advances, deliberated on future perspectives. Clearly, these novel technologies deliver a promising path and beneficial outcomes (Fig. 6). New parallels between different animal models are being discovered, supporting cross-species validations that may improve translational research and ethical issues. The integration of optogenetics and computational systems is allowing for the creation of behavioral paradigms designed to identify the role of specific brain circuits. Accessible machine learning and high-throughput systems are reducing bias and uncovering behaviors previously missed by manual methods. Lastly, this innovative area is dependent on scientists who are equally conversant in computational and biological methods. Thus, the future of behavioral research may provide not only new scientific and technological advances but also opportunities for educating a new generation of interdisciplinary researchers.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neubiorev.2020.11.014.
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