A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: Mapping the way forward

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Abstract

Rodent marble-burying behavior in the marble-burying test (MBT) is employed as a model or measure to study anxiety- and compulsive-like behaviors or anxiolytic and anticomulsive drug action. However, the test responds variably to a range of pharmacological interventions, and little consensus exists regarding specific methodologies for its execution. Regardless, the test is widely applied to investigate the effects of pharmacological, genetic, and behavioral manipulations on purported behaviors related to the said neuropsychiatric constructs. Therefore, in the present review we attempt to expound the collective translational significance of the MBT. We do this by (1) reviewing burying behavior as a natural behavioral phenotype, (2) highlighting key aspects of anxiety and obsessive–compulsive disorder from a translational perspective, (3) reviewing the history and proof of concept of the MBT, (4) critically appraising potential methodological confounds in execution of the MBT, and (5) dissecting responses of the MBT to various pharmacological interventions. We conclude by underlining that the collective translational value of the MBT will be strengthened by contextually valid experimental designs and objective reporting of data.

Keywords Marble-burying test · Validity · Methodology · Review · Anxiety · Obsessive–compulsive disorder · Animal model

Introduction

From a translational point of view, preclinical screening tests can broadly be applied on two levels with respect to neuropsychiatric states: namely, (1) as frameworks with which to study the underlying etiology, neurobiology, and cognitive deficits of specific human conditions, and/or (2) as a means to characterize and quantify the effects of novel pharmacological or behavioral manipulations that are proposed to be of possible benefit in the modeled clinical condition. The accurate and reliable application of such tests relies mainly on three forms of validity—that is, face, construct, and predictive

(Albelda & Joel, 2012a, 2012b; Alonso, Lopez-Sola, Real, Segalas, & Menchon, 2015; Monteiro & Feng, 2016; Willner, 1984). Briefly, *face validity* refers to observable behaviors or signs that seemingly resemble those present in the modeled condition. A screening test or model with robust *construct validity* demonstrates the involvement of neurobiological and/or neurocognitive systems known to be implicit in the modeled condition, whereas *predictive validity* refers to the model's sensitivity to analogous pharmacological or behavioral interventions that are effective in the human condition or to its insensitivity to those that have no demonstrable clinical efficacy. Although few screening tests for psychiatric conditions are founded equally in all levels of validity, a certain degree of overlap between the preclinical and clinical states of being needs to be established, at one or more of the different levels, before the test can be regarded as an instrument appropriate for conducting investigations (Wolmarans, Stein, & Harvey, 2017).

The analysis of marble-burying behavior by rodents is often applied as a preclinical screening test for anxiolytic (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986; Nicolas, Kolb, & Prinszen, 2006; Thomas et al., 2009) and anticomulsive (Egashira et al., 2018; Taylor, Lerch, &

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Chourbaji, 2017; Uday, Pravinkumar, Manish, & Sudhir, 2007; Umathe, Manna, & Jain, 2012) drug action. It is also used as a model to characterize and quantify the purported behavioral manifestations of neuropsychological constructs related to, *inter alia*, posttraumatic stress disorder (PTSD; Kedia & Chattarji, 2014) and Alzheimer's disease (Torres-Lista, López-Pousa, & Giménez-Llort, 2015). However, the marble-burying test (MBT) is characterized by variable responses to a range of pharmacological compounds administered both acutely and chronically (Millan, Girardon, Mullo, Brocco, & Dekeyne, 2002; Sugimoto, Tagawa, Kobayashi, Hotta, & Yamada, 2007; Taylor et al., 2017; Umathe et al., 2012), and little consensus exists regarding the specific methodology to be followed in its execution (Çalışkan et al., 2017), even when the test is applied with respect to a specific condition (Jimenez-Gomez, Osentoski, & Woods, 2011; Sugimoto et al., 2007). Indeed, methodological congruence between different investigations into marble-burying is found only in the application of the number of marbles buried as an indicator of behavioral severity. Considering the unique neurocognitive constructs underlying different mental disorders, such as general anxiety (Mathew, Price, & Charney, 2008), PTSD (Yehuda, 2002), and obsessive-compulsive disorder (OCD; Westenberg, Fineberg, & Denys, 2007), it is perplexing that a seemingly mono-dimensional behavioral phenotype can be applied to mimic and assess the behavioral symptomology of such diverse conditions, albeit within a highly specific context. Although it is not uncommon to apply a specific behavioral measurement in the analysis of behaviors that are believed to mimic different mental disorders—for example, the open field test, in assessments of anxiety (Prut & Belzung, 2003), aggression (Lewis, Gariépy, Gendreau, Nichols, & Mailman, 1994), and social interaction (File & Seth, 2003)—the use of the MBT differs from these tests in that, given the different approaches taken to its application, no clear hypothesis seems to underlie its use within the conceptual and contextual boundaries of a specific disorder. In fact, the realities that the test is applied as a screening tool for specific conditions and drug responses without methodological congruence between laboratories (Dey, Chatterjee, & Kumar, 2016; Gawali et al., 2016) and that it demonstrates inconsistent responses to different treatments (Li, Morrow, & Witkin, 2006; Njung'e & Handley, 1991a) complicate comparisons of the published findings and cloud the translational usefulness of marble-burying as a measure of anxiolytic or anticomulsive drug action.

The purpose of the present article is to provide a critical review of the marble-burying test, first as it is commonly applied as a model in rodents to elucidate the underlying mechanisms that apparently relate to anxiety- and obsessive-compulsive (OC)-like behaviors, and second as a screening test for novel anxiolytic or anti-OC therapies. Because marble-burying is closely related to natural animal behavior (Jirkof, 2014), we will first summarize and appraise the relevant

aspects of normal digging, burrowing, and burying behavior from an ethological perspective. Next we will briefly review key aspects of anxiety and OCD that are of relevance for preclinical research. We will then elaborate on the history and proof of concept of the marble-burying test, followed by a dissection of its experimental application and responses to different treatments. Finally, we will conclude by arguing that in general the marble-burying test—as it has been applied, executed, and reported to this point in time—is of no translational value, and that a contextually standardized approach to the analysis of *aberrant*, but not *inherent* (i.e., occurring as a natural phenotype within the larger population), burying behavior, performed within the context of the preclinical psychiatric research described here, will be pivotal to furthering our understanding of the neurobiological mechanisms underlying clinical anxiety and OCD and their responses to treatment.

Digging, burrowing, and burying as natural behaviors

Digging, burrowing, and burying are core components of the normal behavioral repertoire of rodents (Jirkof, 2014; Layne & Ehrhart, 1970; Pisano & Storer, 1948; Poling et al., 1981; Weber & Hoekstra, 2009). Digging can be regarded as the primary action by which more complex tasks—namely, burrowing or the burying of objects—are achieved, and it refers to the displacement of a substrate using mostly the forepaws (Layne & Ehrhart, 1970). Burrowing—that is, the construction of tunnels for habitation (Adams & Boice, 1981; Sherwin, Haug, Terkelsen, & Vadgama, 2004)—and burying—that is, the displacement of either aversive (De Boer & Koolhaas, 2003) or nonaversive (Poling, Cleary, & Monaghan, 1981) objects beneath any available substrate—thus represent the application of digging to a more specific outcome.

Research into the origins of ethological and laboratory digging has confirmed that digging as a naturally occurring behavior is unaffected by age or sex (Deacon, 2006; Masuda, Ishigooka, & Matsuda, 2000). However, strain variation in digging, burrowing, and burying behavior is common (Deacon, Thomas, Rawlins, & Morley, 2007; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster, Williams, Owens, Geiger, & Dewsbury, 1981). Furthermore, there is evidence that laboratory-reared rats and mice show comparable burrowing activity to their wild-type counterparts when they are afforded living conditions that allow for the complete expression of digging activity (Adams & Boice, 1981; Boice, 1977; Jirkof, 2014; Weber & Hoekstra, 2009). Indeed, digging and burrowing serve analogous purposes across both natural and laboratory settings and are central to rodent survival and social structure (Deacon, 2006; Ebensperger & Blumstein, 2006). Fundamentally, these behaviors are motivationally driven by the needs to store food (Fleming & Brown, 1975;

Jenkins & Breck, 1998), control temperature (Ellison, 1995; Tracy & Walsberg, 2002), facilitate social interaction, nurture and protect young (Denenberg, Taylor, & Zarrow, 1969), and avoid predation (Ebensperger & Blumstein, 2006; Ruffer, 1965; Tracy & Walsberg, 2002). That said, digging and burrowing can also be regarded as a *mandatory behavioral need*, as was demonstrated by Sherwin et al. (2004). Indeed, when studying digging and burrowing behavior in laboratory mice, it has been demonstrated that such behaviors persist in cages that already contain extensive burrow networks. However, although digging and burrowing are natural and persistent under laboratory conditions, even in the offspring of captive-bred animals (Adams & Boice, 1981; Weber & Hoekstra, 2009), such behaviors are subject to modification by a number of factors, including preexposure to the burying substrate—which results in decreased, albeit persistent, burrowing activity (Schultz, 1972)—as well as the burying substrate itself, which can influence the number of burying episodes and the overall measurable digging activity (Layne & Ehrhart, 1970; Webster et al., 1981). Furthermore, gross digging behavior can also be influenced by genetics, even in closely related species, which can exhibit notably different burrow architecture and digging activity (Dudek, Adams, Boice, & Abbott, 1983; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster et al., 1981). Considering that the common standard housing conditions in the majority of rodent housing facilities provide only a thin layer of any given form of bedding material—for example, corn cob (Jimenez-Gomez et al., 2011), wood chips (Deacon et al., 2007), or paper (Burn, Peters, & Mason, 2006)—digging and burrowing often cannot be readily expressed. It can therefore be expected that such behaviors will be expressed to a greater extent following the provision of ample bedding or burrowing substrate (Adams & Boice, 1981; Webster et al., 1981). Furthermore, although the majority of studies that have investigated burying behavior from a novelty—or *neophobic*—perspective have confirmed that anxiety or related concepts of threat/danger both provoke and exacerbate the digging/burying response (De Boer & Koolhaas, 2003; Njung'e & Handley, 1991b; Pinel & Treit, 1978), it is important to emphasize that such behaviors still occur in the absence of such triggers, although possibly they manifest differently (Deacon et al., 2007; Jirkof, 2014; Layne & Ehrhart, 1970; Sherwin et al., 2004; Weber & Hoekstra, 2009).

Although burying behavior, as an intentional outcome of digging, may bear face resemblance to digging or burrowing, burying refers to the *concerted* effort to either cover a particular object with substrate (De Almeida, De Carvalho, Silva, De Sousa, & De Freitas, 2014; De Boer & Koolhaas, 2003; Kinsey, O'Neal, Long, Cravatt, & Lichtman, 2011; Pinel & Treit, 1978; Poling et al., 1981) or displace an object beneath any available substrate by means of *digging in proximity* to it (de Brouwer & Wolmarans, 2018; Gyertyán, 1995). Rodents

have been shown to bury a number of objects, including food (Jenkins & Breck, 1998), marbles (Taylor et al., 2017), live scorpions (Londei, Valentini, & Leone, 1998), rodent chow (either contaminated or uncontaminated; Poling et al., 1981), electrified probes (Treit, 1990), mouse traps (Linfoot et al., 2009), flashing cubes, and air-blasting tubes (Terlecki, Pinel, & Treit, 1979). In light of theories of goal-directed behavior (de Wit & Dickinson, 2009), it can be hypothesized that harmful or noxious objects would elicit a unique burying response, as compared to nonreactive and nonharmful objects. However, investigations into phenotypic differences between the burying behaviors elicited by nonaversive and aversive objects have yielded inconsistent results (Londei et al., 1998; Poling et al., 1981; Terlecki et al., 1979). Collectively, the findings demonstrate that although rodents may initially express increased burying behavior toward potentially harmful, as compared to nonharmful, objects (De Boer & Koolhaas, 2003; Londei et al., 1998; Poling et al., 1981), burying behavior in general is primarily triggered by an investigative drive that is subject to habituation (Broekkamp et al., 1986; de Brouwer & Wolmarans, 2018; Poling et al., 1981; Thomas et al., 2009). It can therefore be concluded that the burying activity engaged in by rodents is a robust, naturally occurring behavior that, although initially modifiable by the nature of the stimulus, is displayed by all animals and is not *dependent* on a contextual trigger.

Fundamentals of anxiety and OCD from a preclinical perspective

Clinical anxiety: a state of abnormal fear processing

Anxiety can be described as a negative state of emotion experienced in anticipation of a forthcoming threat (American Psychiatric Association, 2013; Cryan & Holmes, 2005). However, in contrast to the previous clustering of most anxiety-related conditions into a single diagnostic category based on the presence of anxiety as a common factor (American Psychiatric Association, 2000), the fifth and most recent edition of the *Diagnostic and Statistical Manual of Mental Disorders* separates these conditions into three categories—that is, anxiety disorders, such as generalized anxiety disorder (GAD); trauma and stress-related disorders, such as PTSD; and OC and related disorders, such as OCD and trichotillomania (American Psychiatric Association, 2013). These categories diverge from one another mainly in terms of the underlying fear-provoking trigger, the magnitude of the distress experienced, and the degree of phenomenological and epidemiological overlap between conditions. Although some controversy remains regarding the updated diagnostic categories (Abramowitz & Jacoby, 2015), anxiogenic threats, irrespective of the condition, are of either an internal nature—that

is, born from within the patient, as from concerns about occupational or academic performance—or an external nature—such as confrontation with specific environments or situations, including wide-open spaces or forced social interaction (American Psychiatric Association, 2013). Anxiety is often characterized by transient or long-lasting somatic (e.g., muscle stiffness/weakness, peripheral nervous system activation, fatigue), cognitive (e.g., mental distress that impairs normal function, decreased focus, irritability), and behavioral (e.g., avoidance behavior, disturbed sleep, altered food intake, restlessness) symptoms, which frequently occur in anticipation of possible future threats (American Psychiatric Association, 2013; Nuss, 2015; Ohl, 2003). Furthermore, states of anxiety have the potential to interfere significantly with the daily functioning of a patient. That being said, anxiety *can also be regarded as a normal human response* to threatening or stressing situations, in that short-lived states of anxiety in response to tangible threats are normal (American Psychiatric Association, 2013; Cryan & Holmes, 2005).

Briefly, anxiety arises from the abnormal processing of fear- or stress-provoking stimuli in the limbic system of the brain, which comprises the limbic cortex (insular and cingulate), hippocampus, and amygdala (Martin, Ressler, Binder, & Nemeroff, 2009; Nuss, 2015). Furthermore, the amygdala, as the area responsible for fear expression, aggression, and defensive behavior, consolidates fear memory and receives neuronal and hormonal input from the hippocampus, thalamus, and hypothalamus (Martin et al., 2009). Several neuroendocrine, neurotransmitter, and neuropeptide alterations in these brain regions have been shown to play a role in the manifestation of anxiety. These include attenuated γ -amino butyric acid (GABA)-mediated signaling and bolstered glutamate activity (Meldrum, 1984), and serotonin (5-HT), dopamine (DA), and noradrenalin (NA) have also been implicated (Bandelow et al., 2008). Furthermore, a number of neuropeptides—including cholecystokinin (CCK; Bandelow et al., 2008), neuropeptide-Y (NPY; Tasan et al., 2009), vasopressin (AVP) and oxytocin (Neumann & Landgraf, 2012), Substance P (Santarelli et al., 2001; Tillisch et al., 2012), and corticotropin releasing hormone (CRH; Mathew et al., 2008)—also contribute to the psychopathology of anxiety.

The treatment of anxiety is complicated and is often influenced by the anxiety subtype diagnosed. Although some debate exists, the mainstay approach to the acute treatment of moderate to severe states of anxiety includes the use of compounds that potentiate the effects of GABA—for example, benzodiazepines and barbiturates (Bandelow et al., 2012; Bandelow et al., 2008). However, the focus of long-term management involves chronic administration of antidepressants that target mainly serotonergic and noradrenergic signaling, including selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), serotonin–noradrenalin reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors

(Bandelow et al., 2012; Bandelow et al., 2008). Miscellaneous agents, such as 5-HT_{1A} agonists, antihistamines, atypical antipsychotics, antiepileptic drugs, and beta receptor antagonists, are also used in various instances (Bandelow et al., 2012; Bandelow et al., 2008). Nevertheless, as is also true for OCD (see paragraph 3.2), the efficacy of pharmacotherapeutic intervention in anxiety is, to say the least, suboptimal (Bandelow et al., 2012).

From a preclinical perspective, a distinction needs to be made between the animal models of and behavioral tests for specific conditions. In the case of preclinical neuropsychiatric investigations, animal models refer to robust, valid frameworks that as a whole emulate as closely as possible a holistic picture of the modeled human condition (Geyer & Markou, 1995; van der Staay, 2006). With respect to anxiety, a valid example would be the learned helplessness model, in which rodents not only display behavioral traits akin to those observed in anxiety, but also present with a neurobiological picture resembling that of the clinical condition (Ohl, 2005). On the other hand, a valid test for possible anxiety-like manifestations in any animal, normal or seemingly diseased, refers to a behavioral measure that can accurately identify the phenotype—for example, the elevated-plus maze (EPM; Ohl, 2003). Therefore, animal models of anxiety are dependent on showing that animals are experiencing a certain level of anxiety (Cryan & Holmes, 2005; Ohl, 2003), while the behavioral tests used to measure and characterize such anxiety-like traits should be accurate in doing so. Since rodents do not present with cognitive processing analogous to that of humans, the cognitive aspects of anxiety are often difficult to demonstrate in preclinical models. Therefore, current behavioral tests focus on rodent-specific behavioral parameters in order to recognize, characterize, and assess anxiety-like states—for example, *seemingly abnormal* manifestations of typical rodent behaviors, such as exploration and the avoidance of open and illuminated areas (Cryan & Holmes, 2005; Ohl, 2003), food intake (Ohl, 2003), vigilance and risk assessment (including freezing, stretching, and carefully sniffing unknown objects; Ohl, 2003), and sociability, as well as changes in peripheral nervous system activity (Ohl, 2003). Considering the nonreactive nature of glass marbles, which can be introduced as a novel, unconditioned stimulus (Egashira et al., 2018), it can be hypothesized that the MBT, when applied as a screening test for anxiolytic drug action, could possibly be accurate in measuring anxiety-like manifestations that are best related to a *specific phobia*—that is, neophobia or novelty-induced apprehension (Bruins Slot, Bardin, Auclair, Depoortere, & Newman-Tancredi, 2008; Kedia & Chattarji, 2014; Wolmarans, Stein, & Harvey, 2016). Indeed, phobias are characterized by a disproportionate fear of a specific object or situation that often occurs upon confrontation within the specific fear-related context, resulting in marked anxious responses in human subjects (American Psychiatric Association, 2013). However, as is true

for the MBT's use in research into compulsive-like behavior (Egashira et al., 2018), the differential application of marble-burying both as a model (Njung'e & Handley, 1991b; Jimenez-Gomez et al., 2011) and as a screening test (Kedia & Chattarji, 2014) for anxiety renders its translational usefulness clouded. In fact, neither of these approaches, as currently reported, is valid or appropriate, as we will argue throughout this article.

Apart from the MBT, the shock probe burying test (SPBT) also employs digging behavior as a phenotype of anxious behavior (De Boer & Koolhaas, 2003). As is true for marble-burying (Broekkamp et al., 1986; Jimenez-Gomez et al., 2011; Thomas et al., 2009), shock probe burying also responds to anxiolytic treatment (De Boer & Koolhaas, 2003; Pinel, Treit, Ladak, & MacLennan, 1980). In a comprehensive review of defensive burying (De Boer & Koolhaas, 2003), the authors concluded that along with fight, flight, and freezing, the burying of *aversive* stimuli forms part of the unconditioned defensive repertoire of rodents, representing a form of "active avoidance." Other commonly used, and well validated, tests for anxiety-like manifestations in rodents include the open field test (OFT; Cryan & Holmes, 2005; Ohl, 2003), EPM (Cryan & Holmes, 2005; Ohl, 2003), elevated zero maze (E0M; Cryan & Holmes, 2005), and light–dark test (LDT; Cryan & Holmes, 2005; Ohl, 2003), all four of which *rely on the natural tendency of animals to explore* a novel environment. Here, anxiolytic-like drug properties are indicated by bolstered exploration of central (OFT), open (EPM and E0M), or illuminated (LDT) areas of an arena, as opposed to time spent in the relatively "safe" corner (OFT), enclosed (EPM and E0M), or dark (LDT) spaces. In addition, the EPM and E0M also assess other aspects of anxiety-like behavior—for example, risk-taking behavior—and *specific fears* for an anxiogenic scenario—that is, height. Finally, the social interaction test (SIT; File & Seth, 2003) is used to mimic scenarios of forced social interaction, thereby measuring behaviors related to *fear of and apprehension toward* forced or undesirable social interaction. As such, changes to a number of behavioral parameters, including avoidance, voluntary peer interaction, and investigative social behaviors—for example, following, sniffing, and body contact—are used to recognize anxiolytic-like drug action (Cryan & Holmes, 2005; File & Seth, 2003; Ohl, 2003). Importantly, tests like the SIT provide a platform for testing anxiety-like states by demonstrating sensitivity to anxiolytic drug effects, where the administration of anxiogenic compounds manifests as *reduced* sociability (File & Seth, 2003), thereby adding to the face and predictive validity of the SIT with respect to anxiety. In summary, valid rodent tests for anxiety are accurate in highlighting changes in exploration; avoidance of aversive stimuli, in terms of distal positioning to or covering of the stimulus; reduced risk assessment behavior; decreased sociability; and decreased general activity levels—that is, increased freezing and stationary positioning. With respect to actual anxiety-like behaviors born

from fear of novelty, such behaviors have been shown to abate as a function of repeated exposure (Savy et al., 2015), or even over the course of a single continuous test session (Choleris, Thomas, Kavaliers, & Prato, 2001). Apart from possible changes in exploratory behavior, the MBT does not seem to resemble any such manifestation of anxiety-like behavior, as we will explain later.

Obsessions and compulsions: repetitious, persistent and intrusive

Whereas *obsessions* are described as persistent unwanted images, doubts, or intrusive thoughts that can cause distress (Abramowitz, Taylor, & McKay, 2009), *compulsions* can be understood as repetitive overt or covert behavioral routines that are often expressed in an attempt to alleviate the obsession-driven anxiety (Abramowitz & Jacoby, 2015; American Psychiatric Association, 2013; Veale & Roberts, 2014). However, both obsessions and compulsions can also be diagnosed in the absence of the other characteristic. Indeed, although OCD is no longer considered an anxiety disorder in the most recent version of the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 2013), anxiety, manifesting as a form of psychoneurosis, is accepted to play an important role in OC symptom manifestation (Abramowitz & Jacoby, 2015). However, the anxiolytic relief arising from carrying out compulsions is typically brief and is thought to contribute, via processes of negative reinforcement, to symptom exacerbation (Ahmari, 2016; American Psychiatric Association, 2013). Fundamentally, obsessions and compulsions are attributable to dysfunctional beliefs—that is, a disproportionate sense of importance attached to specific thoughts and feelings (American Psychiatric Association, 2013). These can broadly be grouped within five main symptom clusters: namely, (1) obsessions about contamination and washing compulsions; (2) obsessions concerning harm, together with checking compulsions; (3) obsessions with symmetry, ordering, and counting; (4) repugnant obsessions involving themes of sex, religion, and violence; and (5) hoarding and associated collecting compulsions, recently awarded the status of a separate disorder (Abramowitz et al., 2009; American Psychiatric Association, 2013; Rowsell & Francis, 2015). Importantly, although patients may present with more than one symptom cluster, it is important to note that obsessions and subsequent compulsions mostly develop with respect and in response to specific sets of conditions/stimuli only (American Psychiatric Association, 2013; Rowsell & Francis, 2015). For example, whereas safety-related obsessions are paired with persistent checking compulsions, symmetry obsessions will be associated with excessive ordering compulsions. Patients with OCD therefore function normally under circumstances and in contexts that are unrelated to the triggers of their OC symptoms, and in many cases

they actively avoid their triggers (American Psychiatric Association, 2013). That being said, patients diagnosed with only one symptom dimension are rare (Mataix-Cols, do Rosario-Campos, & Leckman, 2005). Irrespective of the OC cluster, the symptoms can have a negative influence on the normal daily routine of a patient, interfering with the academic, social, and occupational functions of patients, as well as affecting their overall quality of life (Sørensen, Kirkeby, & Thomsen, 2004).

Although different hypotheses have been put forward to explain the symptomology of OCD (Husted, Shapira, & Goodman, 2006), it seems like abnormal regulation of goal-directed behavior may be central to the symptomology of OCD. Thus, the brain areas implicated in OCD are those that translate cognitive planning and experiences into motor behavior, and subsequently mediate goal-directed (viz. reward-related) behavior. These brain areas include the prefrontal cortex, striatum and thalamic nuclei that communicate with each other via different pathways (Evans, Lewis, & Iobst, 2004; Nambu et al., 2000; van den Heuvel et al., 2010). The term “CSTC circuit” (i.e., cortico–striatal–thalamic–cortical circuit) denotes the functional organization of these structures (Stocco, Lebiere, & Anderson, 2010) and consists of both direct (*behaviorally activating*) and indirect (*behaviorally inactivating*) pathways. Although evidence indicates some degree of neuroanatomic variation underlying the various symptom clusters (Rowell & Francis, 2015; Stein et al., 2010)—which may explain some of the variation in treatment response of the different OC phenotypes (McKay et al., 2004)—the CSTC circuit is fundamental in the planning, execution and termination of complex motor behavior and reward-based learning—two major processes that are hypothesized to be dysfunctional in patients with OCD (Stocco et al., 2010). Furthermore, it is believed that there is a bias in favor of the direct thalamus-activating pathway over the indirect thalamus-inhibiting pathway in the basal ganglia of OCD patients, as compared to healthy controls (Saxena & Rauch, 2000). This not only results in an overactive orbitofrontal cortex (OFC), but also increases the activities of both the caudate nucleus and thalamus. The subsequent hyperactivity in the CSTC circuit is believed to be central to the pathological presentation of OCD (Bartz & Hollander, 2006; Saxena & Rauch, 2000).

Certain neurotransmitters have been identified as playing a central role in the pathogenesis of OCD, of which glutamate, GABA, and the monoamines DA and 5-HT have been the most well studied (El Mansari & Blier, 2006; Markarian et al., 2010; Pittenger, Krystal, & Coric, 2006; Sareen et al., 2004). The pharmacological treatment of OCD is based primarily on the use of SSRIs (Abramowitz et al., 2009; Fineberg, 2004; Goddard, Shekhar, Whiteman, & McDougale, 2008), which are typically administered in high doses for extended periods of time (Dougherty, Rauch, & Jenike, 2004; Fineberg, 2004). In fact, the therapeutic onset

of SSRI action can take up to 10 weeks (Goddard et al., 2008). Despite the demonstrable efficacy and tolerability of SSRIs in the treatment of OCD, up to 30% of patients remain treatment-resistant (Fineberg, 2004). In such cases, low-dose antipsychotics, such as risperidone and quetiapine, are often combined with ongoing SSRI treatment in an augmentation strategy (Abramowitz et al., 2009; Dougherty et al., 2004; Fineberg, 2004); however, antipsychotics are typically ineffective when administered as monotherapy (Fineberg, 2004).

Given the abstract and internal nature of obsessions, the presence thereof remains difficult to demonstrate in animal models of OCD (Albelda & Joel, 2012a, 2012b). However, because overt compulsions are often easily observed and characterized, persistent repetitive behaviors form the foundation of animal models of OCD with respect to face validity. In fact, a number of putative and validated animal models of OCD have been described (see Albelda & Joel, 2012a, 2012b; Alonso et al., 2015, for comprehensive reviews in this regard). These include behavioral models, such as spontaneous stereotypy in the deer mouse (Korff, Stein, & Harvey, 2008; Powell, Newman, Pendergast, & Lewis, 1999), excessive nest building (Greene-Schloesser et al., 2011), and compulsive lever pressing (Joel, 2006); pharmacological models, such as quinpirole-induced compulsive-like checking (Szechtman, Sulis, & Eilam, 1998) and 8-OH-DPAT-induced persistent arm preference in a T-maze (Yadin, Friedman, & Bridger, 1991); and genetic models, such as *Sapap3* knockout (KO) mice, which demonstrate excessive self-grooming (Welch et al., 2007). All of these models are, although they differ with respect to the nature of the observable behavior, are congruent with respect to the presentation of persistent and repetitive motor rituals.

With respect to mimicking the human illness as accurately as possible, an animal model of OCD should ideally adhere to a number of criteria. These include repetitive, perseverative, and seemingly inappropriate behaviors that are resistant to change (Alonso et al., 2015; Bechard & Lewis, 2012; Szechtman et al., 2017). Furthermore, cognitive inflexibility, characterized by behaviors without a clear endpoint and that cannot be suppressed indefinitely, such as failure to adapt to altered reward conditions in the signal attenuation test (Joel, 2006), should ideally be present (Joel, 2006; Sohn, Kang, Namkoong, & Kim, 2014). The involvement of abnormal learning processes (Nielen, den Boer, & Smid, 2009) and reliance on habitual rather than goal-directed task performance (Alonso et al., 2015) are also of value. Considering that the expression of compulsions is time-consuming and that it results in significant functional impairment (American Psychiatric Association, 2013; Sohn et al., 2014), animal models of OCD demonstrating such alterations in the normal behavioral routines of subjects may also contribute to our understanding of the intrusive nature of compulsive routines (Wolmarans et al., 2017).

In line with the discussion above, and considering that compulsions are mostly *directed* toward reducing the distress caused by intrusive thoughts related to specific scenarios, we propose that the MBT should be appreciated as characterizing a truly aberrant behavioral phenotype that accurately highlights an animal's persistent, repetitive, and behaviorally inflexible preoccupation and direct interaction with marbles (Njung'e & Handley, 1991b; Wolmarans et al., 2016, 2017), and that incidental or "normal" behaviors that result in the covering of marbles must at all costs be excluded in the assessment of marble-burying behavior. Whether this distinction is appreciated and applied by all investigators is dubious, yet it has major implications for interpreting the presenting behavioral data and the test's translational relevance for human OCD.

In summary, whereas anxiety disorders and OCD are often diagnosed as co-morbid disorders (American Psychiatric Association, 2013; de Mathis et al., 2013), an important distinction between OCD and anxiety disorders is that anxiety-related disorders are typically related to real-world, everyday situations or concerns, whereas obsessions are often of a more abstract and irrational nature (Abramowitz et al., 2009; American Psychiatric Association, 2013). Given the phenotypic presentation of the two conditions alluded to above, it can be hypothesized that an anxious animal will either actively engage to neutralize the anxiety-provoking stimulus or present with passive avoidance and hypervigilance under unfamiliar and/or unconditioned circumstances. However, given the non-reactive and nonharmful nature of the marble stimulus, habituation with respect to both scenarios should develop over time. On the other hand, since OCD is best described by persistent, repetitive, and rigid behaviors, particularly with respect to a specific context, we propose that murine marble-burying behavior, when applied as a screening test for anticomulsive drug action, should preferably be characterized by persistent, repetitive, and voluntary direct interaction with the objects. We will elaborate on this below.

The MBT: a brief history and proof of concept

As we explained earlier, although rodents bury both nonharmful and noxious objects (De Boer & Koolhaas, 2003; Pinel et al., 1980; Pinel & Treit, 1978), burying responses toward the latter group of objects are initially more robust, thus confirming defensive burying as an integral component of the rodent defensive repertoire (for a comprehensive review on the topic, see De Boer & Koolhaas, 2003). Marble-burying, first described within the context of the defensive-burying test, purportedly represents a simplified application of defensive burying, and simply involves the burying of nonaversive marbles in the absence of prior conditioning or training (Poling et al., 1981). However, as is true for defensive burying, the action of marble-burying involves pushing

bedding material toward the marbles with the snout or forepaws or spraying burying substrate over the objects while facing away from the marbles (Gyertyán, 1995). Also, in some cases holes are dug into which marbles are rolled (Gyertyán, 1995). Based on the goal-directed and direct interaction with marbles, such behaviors are typically unique and do not represent normal cage exploration (Gyertyán, 1995; Njung'e & Handley, 1991b).

Marble-burying was initially applied as a screening test for anxiolytic compounds (Broekkamp et al., 1986; Treit, Pinel, & Fibiger, 1981); indeed, the test illustrated accurate predictive validity in that meprobamate, clonazepam and flunitrazepam reduced MB without affecting self-grooming or locomotor responses (normal, nonanxious behaviors; Broekkamp et al., 1986). However, on the basis of observations that rodents will persist in burying nonreactive objects (Gyertyán, 1995; Njung'e & Handley, 1991b), including familiar food pellets (Poling et al., 1981; Thomas et al., 2009), the proposed anxiety-like involvement in marble-burying behavior, and thus also the fundamental value of the MBT, has been questioned. Although it is true that the drug challenges introduced in numerous investigations have yielded results that largely agree with anxiolytic responses (Broekkamp et al., 1986; Bruins Slot et al., 2008; Kinsey et al., 2011), additional behavioral investigations have indicated a clear lack of anxiety-like involvement in the marble-burying response, whereas a number of nonanxiolytic compounds, such as haloperidol, also reduce marble-burying behavior (Bruins Slot et al., 2008; Matsushita et al., 2005; Nicolas et al., 2006). In fact, it has been shown that marble-burying persists over repeated exposure, even when avoidance of the marbles is possible and after animals have been habituated to the presence of marbles in their home cage environments for extended periods (Njung'e & Handley, 1991b; Thomas et al., 2009). Moreover, the facts that marble-burying correlates poorly with the outcomes of other classical experimental tests of anxiety (Sanathara et al., 2018; Savy et al., 2015; Thomas et al., 2009; although see also Greene-Schloesser et al., 2011) and that it is subject to significant between-strain variation (Angoa-Pérez, Kane, Briggs, Francescutti, & Kuhn, 2013; Egashira et al., 2013; Nicolas et al., 2006; Thomas et al., 2009) further suggest that marble-burying as it is normally carried out in the lab—that is, not based on individual differences in behavior, but rather to characterize group differences in *supposed* abnormal burying behavior, mostly following drug intervention—represents an inherent, rather than a neophobic or anxiety-related, behavioral phenotype. As such, the MBT has since been employed to model the purported behavioral manifestations of OCD—that is, seemingly purposeless repetition (Egashira, Harada, et al., 2007; Gaikwad, Parle, Kumar, & Gaikwad, 2010; Iijima, Kurosu, & Chaki, 2010; Taylor et al., 2017; Umathe, Bhutada, Dixit, & Shende, 2008; Umathe et al., 2012); in most cases, however, this approach

is also unjustified, as will be explained. However, it must be emphasized that it is possible to induce excessive marble-burying behavior by using known anxiogenic interventions, such as restraint-induced stress (Kedia & Chattarji, 2014). In this regard, putative anxiolytics and anticomulsive compounds can be identified on the basis of their effects on *induced* burying behavior; this is because a direct association can be made between an elicited anxiogenic or compulsive-like response and favorable drug action. That said, most anxiety- and compulsivity-related investigations that have employed marble-burying as a measure of behavioral severity have applied naturalistic, noninduced burying activity in its own right as a means to identify and characterize anxiolytic and anticomulsive drug action (Broekkamp et al., 1986; Bruins Slot et al., 2008; Londei et al., 1998; Umathe et al., 2012), an approach that is inherently flawed.

A methodological review of the MBT

The oft-reported methodology for the MBT is simple and based largely on the study design employed by Broekkamp et al. (1986). Briefly, the test involves the placement of any number of marbles (usually between 4 and 25, depending on the zone configuration of the marble-burying arena; see paragraphs 5.2 and 5.5; Çalişkan et al., 2017) gently onto the surface of a layer of bedding material (normally no thicker than 5 cm). However, whereas Broekkamp et al. (1986) placed the marbles in close contact with one another centrally in the cage, most recent investigations have employed an experimental configuration in which marbles were spaced evenly throughout the arena (one-zone; 1Z; Egashira et al., 2018; Gawali et al., 2016; Millan et al., 2002) or in one section of the arena only (two-zone; 2Z; Gyertyán, 1995; Nicolas et al., 2006; Njung'e & Handley, 1991b; Torres-Lista et al., 2015). Occasionally, marbles are placed around the perimeter of the burying cage, as well (Chaki et al., 2003; Taylor et al., 2017; Young, Batkai, Dukat, & Glennon, 2006), resembling another example of a two-zone setup. Subsequently, subjects are introduced to the marble-containing arenas for up to 30 min (Çalişkan et al., 2017) and are allowed voluntary interaction with the environment. The number of marbles that have been buried is then counted by an observer, who is often blind to the treatment status of the subjects (Angoa-Pérez et al., 2013; Egashira et al., 2018; Millan et al., 2002). Importantly, since the majority of investigations do not distinguish between the specific behavioral patterns resulting in marbles being lowered into the burying substrate (Egashira, Okuno, Abe, et al., 2008; Gaikwad & Parle, 2011; Gomes, Casarotto, Resstel, & Guimarães, 2011; Harasawa, Ago, Itoh, Baba, & Matsuda, 2006; Honda, Kawaura, Soeda, Shirasaki, & Takahama, 2011; Li et al., 2006; Matsushima, Shirota, Kikura-Hanajiri, Goda, & Eguchi, 2009; Shimazaki, Iijima,

& Chaki, 2004; Umathe et al., 2012; Yamada et al., 2002), the term “buried” is generally applied to indicate the number of *covered* marbles. Although the reported methodologies always refer to the duration of exposure as well as the number of marbles and the burying substrate used, several experimental variables are subject to modification, either intentionally or not. Such interinvestigation differences, although they may often have meaningful implications for interpretation of the findings, are not always declared. Therefore, the following paragraphs will summarize key aspects of these variables and explain their fundamental meanings within the context of behavioral investigations.

Burying substrate and its relation to marble size

Substrates commonly used include corn cob (Supplementary Fig. 1a; Angoa-Pérez et al., 2013; Jimenez-Gomez et al., 2011; Thomas et al., 2009), sawdust (Supplementary Fig. 1b; Bruins Slot et al., 2008; Dixit et al., 2014; Harasawa et al., 2006; Krass, Rünkorg, Wegener, & Volke, 2010), wood chips (Supplementary Fig. 1c; Llaneza & Frye, 2009; Londei et al., 1998; Saadat, Elliott, Colado, & Gree, 2006; Thomas et al., 2009), wood shavings (Supplementary Fig. 1d; Poling et al., 1981), river sand (Supplementary Fig. 1e; de Brouwer & Wolmarans, 2018), and Sani-chips (Supplementary Fig. 1f; Kinsey et al., 2011; Thomas et al., 2009; Young et al., 2006). However, detailed descriptions of the respective burying substrates, including mass per volume, particle size, and manner of placement—namely, compacted or not—are almost never reported (Casarotto, Gomes, Resstel, & Guimarães, 2010; Egashira, Okuno, Abe, et al., 2008; Egashira, Okuno, Harada, et al., 2008; Iijima et al., 2010; Umathe et al., 2012). Indeed, such details are important for a number of reasons. Due to the sparse and light nature of burying substrates such as sawdust (pine, weighed and calculated at 0.17 g/cm³; de Brouwer & Wolmarans, 2018) and wood shavings (pine, weighed and calculated at 0.07 g/cm³; de Brouwer & Wolmarans, 2018), marbles simply placed gently on the surface of these substrates may appear from the outset to be covered to a depth of at least two-thirds of their size, when compared to more dense substrates (de Brouwer & Wolmarans, 2018; Supplementary Fig. 2a; see also Paragraph 5.6). Even if marbles are placed with care, any disturbance of the surrounding bedding material by any form of exploratory activity by the animal may result in marbles settling deeper, or even beneath, the substrate. Since most investigations do not report the use of video tracking, except for purposes of locomotor activity (LMA) tracking (Nicolas et al., 2006), endpoint quantification of the number of marbles buried may be a caveat in the interpretation of data. In contrast, denser substrates with a higher mass-per-volume ratio—for example, corncob (weighed and calculated at 0.38 g/cm³; de Brouwer & Wolmarans, 2018) or fine river sand (Supplementary Fig. 2b; weighed and

calculated at 1.65 g/cm^3 ; de Brouwer & Wolmarans, 2018)—are generally more resistant to the effects of normal exploration, and therefore may be better suited as appropriate substrates in which to carry out the MBT (Supplementary Fig. 3a vs. 3b; Angoa-Pérez et al., 2013; de Brouwer & Wolmarans, 2018; Jimenez-Gomez et al., 2011).

Moreover, considering that the number of marbles used in the execution of the MBT typically varies from 4 to 25 (Çalışkan et al., 2017; Gaikwad et al., 2010; Krass et al., 2010; Schneider & Popik, 2007; Sugimoto et al., 2007; Uday et al., 2007; Umathe et al., 2008), and that the most common criterion applied to quantify buried marbles is equated to two-thirds of the marble being covered, the particle size of the burying substrates is an equally important variable. For instance, whereas a 15-mm marble used in our laboratory weighs 5.6 g, a 20-mm or 23-mm glass marble of similar density would weigh approximately 13.26 or 20.16 g, respectively. This is important for two reasons. First, it is evident that marbles of a greater mass would settle to the bottom of the testing arena more quickly than marbles of a lower mass, especially when placed in cages containing burying substrates of a sparse nature—for example, sawdust (Supplementary Fig. 1b) or wood shavings (Supplementary Fig. 2a)—which are subject to disturbance by any routine movement of the animals during the test session (de Brouwer & Wolmarans, 2018). Second, smaller marbles (e.g., 10 mm) introduced into an arena prepared with burying substrates of a larger particle size—for example, wood shavings (Supplementary Fig. 2a; average diameter of 8 mm)—may complicate the quantification of the number of marbles buried, as compared to cages prepared with larger marbles in relation to the substrate particle diameter (Supplementary Fig. 2b). This may include scenarios in which either smaller marbles are placed in cages with burying substrates with a small particle diameter (e.g., 10-mm marbles in cages prepared with corncob; particle $\phi = 4 \text{ mm}$; Supplementary Fig. 1a) or larger marbles are placed in cages containing burying substrates with a larger particle size (e.g., 15-mm marbles in cages fitted with sawdust; particle $\phi = 8 \text{ mm}$). This is true because the commonly applied two-thirds-covered criterion to quantify the number of “buried” marbles is based on visual observation of the testing arena. In line with this argument, Gyertyán (1995) investigated the effect of marbles on observable digging behavior. The findings there indicated that digging behavior in lighter and sparser substrates—for example, sawdust—occurred similarly in cages containing and not containing marbles. These findings were replicated by Thomas et al. (2009). Importantly, Gyertyán also demonstrated that the covering of marbles with bedding material from digging bouts occurred incidentally because of general digging activity, rather than as a result of marble-directed activity, and that such digging behavior was by no means either triggered or bolstered by the presence of the marbles. This finding possibly elucidates why marbles spaced evenly throughout the

cage (Njung’e & Handley, 1991b), instead of in close proximity of one another in the center of the cage (Broekkamp et al., 1986), seem to bolster burying activity findings.

Zone configuration—a question of choice

As we alluded to earlier, the MBT can be carried out with marbles spaced evenly throughout the entire arena (Badgujar & Surana, 2010; Bruins Slot et al., 2008; Kinsey et al., 2011) or placed in one section of the arena only (Llaneza & Frye, 2009; Nicolas et al., 2006; Njung’e & Handley, 1991b; Savy et al., 2015; Schneider & Popik, 2007; Thomas et al., 2009). A core concept of the MBT when applied as a measure of anxiety is that animals unconditioned to the testing paradigm may experience neophobia-related anxiety when confronted with marbles for the first time, thereby invoking either active burying or passive avoidance behavior (Bruins Slot et al., 2008; Kinsey et al., 2011). This idea was mostly strengthened by early findings demonstrating the efficacy of anxiolytic drugs in reducing marble-burying activity (MBA) without affecting other behaviors (Broekkamp et al., 1986), whereas numerous investigations have subsequently aimed to examine whether novelty-induced anxiety is indeed a trigger for marble-burying behavior (Gyertyán, 1995; Nicolas et al., 2006; Njung’e & Handley, 1991b; Thomas et al., 2009). To appropriately characterize anxiety-like responses in the MBT, a 2Z paradigm is ideal, since the majority of behaviors observed in the 1Z paradigm are related to exploration and investigation of a novel environment (de Brouwer & Wolmarans, 2018). Furthermore, as opposed to 1Z setups, the behaviors in a 2Z paradigm more appropriately reflect goal-directed interaction while also being reflective of definite avoidance behavior. Interestingly, with few exceptions (see, e.g., Schneider & Popik, 2007), most 2Z investigations have failed to demonstrate passive avoidance of marbles (Broekkamp et al., 1986; de Brouwer & Wolmarans, 2018; Kaehler, Singewald, Sinner, & Philippu, 1999; Nicolas et al., 2006; Njung’e & Handley, 1991b; Thomas et al., 2009). That said, these findings also do not indicate active neutralizing interaction with marbles, in that animals often spend equal time in both zones of the arena, regardless of the duration of the experiment, even if the two zones are separated by a divider (Nicolas et al., 2006; Njung’e & Handley, 1991b; Savy et al., 2015; Thomas et al., 2009; Torres-Lista et al., 2015).

However, considering that *externally provoked* marble-burying behavior may be a valid means to assess putative anxiolytic responses, Kedia and Chattarji (2014) showed that mice stressed by acute immobilization buried more marbles following experience of the stressor than did nonstressed counterparts, whereas Llaneza and Frye (2009) demonstrated a positive correlation between increased MBA and increased vigilance/immobility behavior in a shock-induced conditioned fear paradigm. Furthermore, significantly bolstered marble-

burying was also found in prior-stressed animals (Dey et al., 2016). Increased marble-burying has also been reported in animals injected with diphtheria toxin (Sanathara et al., 2018). Since the investigation by Kedia and Chattarji employed MBA to measure the manifestations of *induced* anxiety (that is to say, as a measure of induced anxiety) in animals, it cannot be concluded that the presence of marbles in test arenas per se contributed to the experienced level of anxiety; rather, it may be an accurate predictor of anxiogenic manipulation, albeit variably so (cf. Kedia & Chattarji, 2014, vs. Nicolas et al., 2006; Njung'e & Handley, 1991b). It can therefore be considered that although evidence indicates that the anxious state of an animal may affect its response toward the presence of marbles, such association remains unlikely where the marbles are introduced as anxiogenic stimuli. In fact, many researchers have concluded that anxiety and associated concepts of novelty are *not driving or reinforcing factors* for marble-burying behavior, but that such behaviors *in the majority of animals* are born from a need to investigate novel surroundings (Gyertyán, 1995; Masuda et al., 2000; Nicolas et al., 2006; Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009; Wolmarans et al., 2016).

Although the literature concerning animal models of OCD has since repositioned the MBT more as a measure of compulsive-like, instead of anxiety-like, behavior (Albelda & Joel, 2012a, 2012b; Alonso et al., 2015; Thomas et al., 2009), investigations applying the MBT in anxiety-related studies are still performed (Nicolas et al., 2006; Saadat et al., 2006); these may also benefit from employing a 2Z configuration. With respect to the MBT, and in line with OC theory (Alonso et al., 2015), an animal expressing compulsive-like behavior should repetitively and persistently engage in burying behavior, even if presented with a choice not to engage in such activity. If an animal therefore chooses to avoid exposure to the marbles, compulsive-like repetition should be excluded, hence undermining the application of the test under such specific circumstances as a measure of anticomulsive drug action (Wolmarans et al., 2016).

To habituate or not

Since marble-burying behavior may partly be driven by an inherent need for investigation, the novelty of burying substrates may trigger natural exploratory activity, in the form of digging and burrowing, and may therefore influence the number of marbles being covered (Gyertyán, 1995; Thomas et al., 2009). Although bolstered burying activity under novel circumstances has been demonstrated previously (Schultz, 1972), an influence of novelty-induced anxiety with respect to unfamiliar bedding or marbles has also largely been excluded (Gyertyán, 1995; Thomas et al., 2009), providing further support that marbles are often covered as a coincidental effect of normal exploratory behaviors (de Brouwer & Wolmarans,

2018; Gyertyán, 1995; Njung'e & Handley, 1991b; Thomas et al., 2009).

However, to exclude the possible effects of novel cage exploration on burying outcomes, it is important to consider adequate habituation with the burying substrates before the onset of behavioral analysis. This may be more applicable for anxiety than for compulsivity-related studies. This is because in compulsivity studies, animals should be exposed over the course of repetitive trials instead of a single trial (de Brouwer & Wolmarans, 2018; Gyertyán, 1995; Njung'e & Handley, 1991b; Taylor et al., 2017; Thomas et al., 2009; Wolmarans et al., 2016); hence, habituation is introduced coincidentally in the experimental design. In fact, when the test was repeated on up to five consecutive days with the same subjects, no significant differences in MBA were found across several different investigations (Gyertyán, 1995; Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009; Wolmarans et al., 2016). Also, it has been shown that marble-burying behavior remains unaltered even when the test is repeated many times over the course of a single day (Njung'e & Handley, 1991b; Thomas et al., 2009). In line with this, performance on the MBT is unaltered even after habituating the subjects to marbles in a home cage environment for a 5- to 21-day period (Njung'e & Handley, 1991b; Poling et al., 1981; Thomas et al., 2009). Furthermore, in studies relating to anxiety, the point of departure is to introduce a novel, unconditioned stimulus in the form of marbles. It is important, therefore, to exclude the possible effects of other novelty factors, such as the burying substrate, on marble-burying performance (Casarotto et al., 2010; Gawali et al., 2016; Taylor et al., 2017; Umathe et al., 2012).

Locomotor performance

Since the burying of marbles entails physical motor activity, an important aspect of the MBT that needs consideration in the study design and the interpretation of findings is the inherent LMA of the subjects being tested. Although this is frequently reported alongside MBT results, there are examples in which LMA assessments have not been discussed (Angoa-Pérez et al., 2013; Kedia & Chattarji, 2014). Due to the central acting agents often modifying the normal locomotor abilities of subjects (Kinsey et al., 2011; Lynch, Castagné, Moser, & Mittelstadt, 2011), it is important to distinguish purported anxiolytic and anticomulsive drug action from incidental inhibition of LMA, which may manifest in practical terms as altered MBA (Nicolas et al., 2006). In fact, findings showing drug-decreased MBA paired with markedly suppressed LMA are often discarded as false-positive data (Kinsey et al., 2011; Njung'e & Handley, 1991a). Investigations into MBA that have also reported results from locomotor assessments have employed several approaches, including separate analyses of LMA (Krass et al., 2010; Millan et al., 2002; Saadat et al., 2006) and simultaneous recording of marble-burying and LMA (Egashira et al., 2018;

Egashira, Okuno, Matsushita, et al., 2008; Jimenez-Gomez et al., 2011; Matsushita et al., 2005; Nicolas et al., 2006). Furthermore, LMA assessments may employ either the same subjects tested for marble-burying behavior (Egashira et al., 2013; Schneider & Popik, 2007; Umathe, Vaghasiya, Jain, & Dixit, 2009) or a separate group exposed to treatment regimens analogous to that of the group tested for burying behavior (Gaikwad et al., 2010; Saadat et al., 2006; Uday et al., 2007). Considering that marble-burying behavior may involve preoccupation with objects, it is possible that simultaneous measurements of burying and locomotion may yield inappropriate results, since animals engaging in burying activity may travel shorter overall distances than animals engaging in normal exploratory routines (de Brouwer & Wolmarans, 2018). Although this remains to be established, it is an aspect that needs careful consideration in the interpretation of data obtained from marble-burying investigations.

Arena size

Significant variation exists across investigations with respect to the size of the arena employed (Broekkamp et al., 1986; Casarotto et al., 2010; Egashira, Harada, et al., 2007; Millan et al., 2002; Njung'e & Handley, 1991b; Thomas et al., 2009; Uday et al., 2007; Umathe et al., 2008; Wolmarans et al., 2016). Furthermore, since both rats and mice are employed in marble-burying investigations, it would be expected that appropriately larger arenas would be employed for rats, as compared to those used in mouse studies. However, no clear guidelines exist, with rat arenas ranging from 35 × 25 × 19 cm (Poling et al., 1981) to 45 × 24 × 21 cm (Llaneza & Frye, 2009) and 47 × 27 × 15 cm (Schneider & Popik, 2007). Important to note is that the latter two studies employed the two-zone test, effectively utilizing half of the floor space for marble-burying. It is perhaps with respect to the arenas used for studies relating to mouse burying behavior that the greatest variation is observed; in some cases, the arenas employed have been of similar dimensions, or even larger than, those employed in rat studies. These sizes range from 23 × 17 × 14 cm (25 marbles; Broekkamp et al., 1986) and 38 × 32 × 28 cm (25 marbles; Casarotto et al., 2010) to 45 × 60 × 25 cm (24 marbles; Badgujar & Surana, 2010). Arena size is important because the “density” of marbles spread across the bedding material surface, depending on the number of marbles used, would differ between smaller and larger arenas. Between-laboratory differences in this regard may significantly influence behavioral performance and the subsequent interpretation of data, especially in experimental paradigms employing a 1Z setup (Badgujar & Surana, 2010; Bruins Slot et al., 2008; Dixit et al., 2014). In experiments in which the possibility to avoid exposure to marbles is undesirable (1Z condition), large between-marble spaces may undermine the purpose of the investigation, as is evident when comparing the ratios of floor space to marbles reported in the literature. Higher marble

densities of 21.2 cm²/marble (Bruins Slot et al., 2008) or 23.8 cm²/marble (Dixit et al., 2014) stand in stark contrast to lower densities of 112.5 cm²/marble (Badgujar & Surana, 2010) or 75 cm²/marble (Saadat et al., 2006), all of which have been employed in a 1Z test configuration. Furthermore, a densely spaced marble grid may be overly subject to incidental covering of the marbles, resulting from indiscriminate disturbances of the burying substrate during nonspecific behavioral routines (de Brouwer & Wolmarans, 2018; Gyertyán, 1995; Njung'e & Handley, 1991b). That said, whereas arena size is an important factor for consideration in the defensive-burying test, in which the extent of shock probe burying is *reduced* by larger rectangular arenas (Cueto-Escobedo, Contreras, Bernal-Morales, Guillen-Ruiz, & Rodríguez-Landa, 2013) and *induced* by circular arenas (Cueto-Escobedo et al., 2013; Davis, Moore, Cowen, Thurston, & Maggio, 1982), few investigations have explicitly analyzed the influence of arena size in the MBT. In one such investigation, Poling et al. (1981) hypothesized that a restricted test arena could potentially force subjects to interact with marbles or other “bury”-inducing objects. They tested this hypothesis by examining the burying of regular, uncontaminated food pellets and marbles in a smaller- as well as a larger-than-home-cage-sized arena. However, objects were buried equally in both paradigms, suggesting that restricting the arena size, and thereby forcing interaction between the subject and the marbles, neither triggers nor bolsters burying activity.

A matter of counting—observers and scoring criteria

Because scoring in the MBT is *always* performed manually, three important between-laboratory variables that differ in assessments of burying activity are (1) the number observers (either blind or nonblind); (2) the criteria to determine what constitutes a buried marble, be it one-half (Kinsey et al., 2011; Schneider & Popik, 2007; Taylor et al., 2017; Thomas et al., 2009), two-thirds (Broekkamp et al., 1986; Egashira, Harada, et al., 2007; Egashira, Okuno, Harada, et al., 2008; Gawali et al., 2016; Harasawa et al., 2006; Iijima et al., 2010; Jimenez-Gomez et al., 2011; Kedia & Chattarji, 2014; Millan et al., 2002; Nicolas et al., 2006; Takeuchi, Yatsugi, & Yamaguchi, 2002; Uday et al., 2007), or completely (Ichimaru, Egawa, & Sawa, 1995; Torres-Lista et al., 2015) covered; and (3) whether video recordings of the test sessions are made, to verify the counting results and examine specific behaviors (Jimenez-Gomez et al., 2011; Kedia & Chattarji, 2014; Umathe et al., 2012). Since the counting of marbles remains a subjective visual inspection of the test cage, there exists room for observers to make interpretations of what may or may not constitute a buried marble according to the chosen criteria, which may further be complicated by substrates of a sparse nature (see paragraph 5.1; de Brouwer & Wolmarans, 2018). To this end, methods to eliminate such inherent biases may be useful, as in the studies of Angoa-Pérez et al. (2013)

and Kinsey et al. (2011), in which two observers counted marbles and data were accepted only when they met a certain agreement criterion or scores were averaged between observers. Such improvements to the interpretation of experimental results have been highlighted in initiatives such as the ARRIVE guidelines, which aim to improve the quality of animal research reporting while simultaneously attempting to optimize ethical concerns (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

Effect of sex, species, strain, and genes

Sex is an important factor for consideration regarding marble-burying performance. Two-thirds of studies have employed male subjects (Çalışkan et al., 2017), 8% of the marble-burying investigations to date have employed only females, and the remainder have operated without sex bias, or with no reference to sex at all (Badgujar & Surana, 2010; Çalışkan et al., 2017; Njung'e & Handley, 1991a, 1991b; Taylor et al., 2017; Yamada et al., 2002). With respect to the possible influences of sex on marble-burying behavior, the oestrous cycle has been shown to have at least some influence on burying activity. In fact, it has been demonstrated that marble-burying is bolstered during the rat metoestrus and reduced during proestrus, which correlate with suppressed and elevated sex hormone levels, respectively (Schneider & Popik, 2007). However, this effect was not observed in *all* subjects in that investigation. Furthermore, Llaneza and Frye (2009) found that both MBA and vigilant/avoidant behaviors were minimized during the oestrous phase, in line with the observations of Schnieder and Popik. In addition, it was shown that the administration of exogenous progesterone and estradiol to noncycling ovariectomized dams attenuated anxiety and MBA in a fashion similar to cycling dams during the oestrous phase (Schneider & Popik, 2007). Also, Schnieder and Popik demonstrated that the naturally increased MBA that occurred during metoestrus was attenuated by progesterone administration. It is therefore entirely possible that the ovarian cycle is a potential confounder of marble-burying results. That said, the importance of using both sexes in OCD studies has been highlighted (Albelda & Joel, 2012b; Taylor et al., 2017), whereas the rationale for a study to exclude either of the sexes needs careful consideration and motivation, as is noted in the ARRIVE guidelines (Kilkenny et al., 2010). This, by extension, also applies to anxiety testing, since ovarian cycling has been shown to modulate test results (Frye, Petralia, & Rhodes, 2000), and even drug response in anxiety investigations (Regenass, Möller, & Harvey, 2018).

The defensive-burying response, from which the MBT is methodologically derived (Poling et al., 1981), was first demonstrated in rats. Since the publishing of the first articles in which marble-burying was applied in rodents, a large number of mouse and rat strains have been employed, including Swiss Albino (Gaikwad et al., 2010; Jimenez-Gomez et al., 2011;

Uday et al., 2007; Umathe et al., 2008; Umathe et al., 2012), C57Black/6J (Casarotto et al., 2010; Gomes et al., 2011; Kedia & Chattarji, 2014; Kinsey et al., 2011; Krass et al., 2010; Nicolas et al., 2006; Thomas et al., 2009), ICR (Egashira, Harada, et al., 2007; Egashira, Okuno, Abe, et al., 2008; Egashira, Okuno, Harada, et al., 2008; Egashira, Okuno, Matsushita, et al., 2008; Iijima et al., 2010; Matsushita et al., 2005; Shimazaki et al., 2004; Sugimoto et al., 2007), NMRI (Bruins Slot et al., 2008; Gyertyán, 1995; Millan et al., 2002), MF1 (Njung'e & Handley, 1991a, 1991b), ddY (Abe, Nakai, Tabata, Saito, & Egawa, 1998; Egashira et al., 2013; Honda et al., 2011), and most recently, the deer mouse (de Brouwer & Wolmarans, 2018; Wolmarans et al., 2016). Wistar (Schneider & Popik, 2007) and Long-Evans/hooded (Llaneza & Frye, 2009; Poling et al., 1981) rats have also been used, as well as a number of miscellaneous strains (Çalışkan et al., 2017; please also refer to the [supplementary tables](#)). Variations in digging activity based on genetics and the typical natural environments of species have extensively been reported in the literature (Dudek et al., 1983; Layne & Ehrhart, 1970; Weber & Hoekstra, 2009; Webster et al., 1981). To investigate the influence that strain may have on marble-burying and avoidance behaviors, Nicolas et al. (2006) investigated the burying-related activities of three strains of mice—that is, C57BL/6J, BALB/c, and CBA/J. Indeed, it was reported that BALB/c mice presented with the lowest level, and CBA/J and C57BL/6J with higher levels of burying activity, congruent with and supporting earlier findings (Dudek et al., 1983). Subsequently, Nicolas et al. selected C57BL/6J and CBA/J for further study with respect to neophobic responses in a 2Z setup. Here, no significant difference in the numbers of marbles buried between the two strains was found; however, C57BL/6J mice spent markedly less time on the marble-containing side than did CBA/J mice. Since the C57BL/6J mice, and not the other two strains, presented with both avoidance anxiety and superior MBA, their behavior potentially presents with robust face validity for modeling anxiety-like manifestations in the MBT (Nicolas et al., 2006). Unfortunately, the test was carried out only once in all subjects, and it is therefore not clear whether the seemingly neophobic response in C57BL/6J mice would have habituated over time. Thomas et al. (2009) later expanded on strain differences by testing ten strains of mice with respect to a number of anxiety-related behaviors, including marble-burying behavior in a 1Z paradigm. However, in this study, although the findings concerning CBA/J mice were largely congruent with those of Nicolas et al., C57BL/6J mice buried fewer marbles than did many of the other strains (Thomas et al., 2009). When considering how rodent strain effects may relate to OCD, it is interesting that Korff et al. (2008) demonstrated significantly greater stereotypic behavior in deer mice, a recognized animal model of OCD, than in C57BL mice, which also concurs with the aforementioned findings

of different expressions of purported OC-like behaviors by different strains. The differences in evinced anxiety and stereotypic behavior observed in these two strains further highlight the importance of separating anxiety- and OC-related behaviors in a test or model. This is, of course, where the MBT receives its harshest criticism. However, it is important to note that Nicolas et al. (2006) first assessed burying activity in a 1Z paradigm, whereby interaction with the marbles was forced, and that they subsequently observed avoidance behavior in a 2Z paradigm. The findings of Thomas and Nicolas are therefore congruent with respect to the neophobic trait reported in C57BL/6J mice, highlighting the presented evidence that the MBA observed in most strains, except for C57BL/6J, is poorly correlated with other conventional tests of anxiety, such as open field exploration and the light–dark avoidance test (Cryan & Holmes, 2005; Ohl, 2003). Although these results contradict some findings from other laboratories (Greene-Schloesser et al., 2011), they highlight the significance of between-laboratory methodological differences and the influence they may have on the reporting and interpretation of data.

In addition to different strains being used, a number of genetically modified strains have also been characterized with respect to burying behavior. Since these studies are numerous and mostly included the MBT only as one of a number of behavioral experiments (Angoa-Pérez et al., 2013; Balemans et al., 2010; Burne, McGrath, Eyles, & Mackay-Sim, 2005; Duangdao, Clark, Okamura, & Reinscheid, 2009; Egashira, Tanoue, et al., 2007; Gavioli, Rizzi, Marzola, Zucchini, & Regoli, 2007; Lähdesmäki et al., 2002; Mosienko et al., 2012; Sanathara et al., 2018; Shmelkov et al., 2010; Tasan et al., 2009; Yamada et al., 2002), we will only highlight some key findings here. Tryptophan hydroxylase 2 (TPH2) knock-out (KO) mice, which present with no detectable brain levels of 5-HT, demonstrate bolstered MBA in a 1Z paradigm as compared to their wild-type counterparts (Angoa-Pérez et al., 2013). In contrast, mice lacking the vitamin D (VDR; Burne et al., 2005) and vasopressin-1a (V1a; Egashira, Tanoue, et al., 2007) receptors have been demonstrated as burying fewer marbles than their respective wild-type controls, indicating that MB performance is indeed affected by genetic manipulation in addition to the more common pharmacological manipulations carried out in the test. In this regard, the adrenergic system has also been implicated, since α_2 -adrenergic receptor KO mice bury markedly fewer marbles than their wild-type counterparts (Lähdesmäki et al., 2002). Interestingly, and considering that the MBT may be accurate in mimicking anxiogenic responses, knockout of the nociception/orphanin FQ peptide receptor (NOP), which normally produces anxiolytic effects when stimulated, caused an anxious behavioral phenotype in the EPM and light–dark tests, but not in marble-burying and several other classic tests of anxiety. This led the authors to conclude that the NOP

receptor possibly plays a role in the *modulation* of anxious behaviors, albeit on an exceedingly complex level (Gavioli et al., 2007). Finally, neuropeptide Y receptors, specifically the Y2 and Y4 receptors, seem to modulate anxiety-like behavior, in that Y2- and Y4-KO mice have been demonstrated to present with anxiolytic responses in the MBT as well as in other anxiety tests (Tasan et al., 2009). Mice lacking the melanin-concentrating hormone receptor (MCHR) also display increased MBA (Sanathara et al., 2018), findings that were further corroborated by pharmacological interventions in the same study. Together, these findings suggest that genetic KO models do indeed provide an interesting avenue for further research, but that the aforementioned genetic KO investigations are subject to many of the methodological constraints highlighted throughout this review.

As we have illustrated in the preceding paragraphs, sex, species, strain, and genes all have profound effects on digging behavior as a whole, and thus, each of these parameters requires careful consideration with respect to analyses of marble-burying performance. Indeed, findings reported following the use of different sexes or strains may not be comparable between laboratories, due to apparent differences in the inherent behaviors of the respective subjects. Depending on the application of the MBT, strains that exhibit robust burying/digging behavior and/or neophobic behavior may be ideal.

To summarize this section, it is evident that a number of methodological differences characterize the execution of the MBT. Furthermore, as we explained in the preceding paragraphs, the importance of these differences in the presentation, analysis, and interpretation of findings related to anxiety- and compulsive-like activity cannot be overstated. Thus, the question arises of how such an apparently mono-dimensional and often inherent behavior can be applied and appraised as the core symptom of a number of cognitive constructs, without consideration of the methodological confounds discussed above. This question will now be discussed from a pharmacological perspective.

Responses of marble-burying to pharmacological manipulation

Given its application in a wide range of translational frameworks, the MBT has been challenged with a wide range of pharmacological compounds. Indeed, if we consider Tables 1, 2, and 3, marble-burying has demonstrated varying responses to a number of interventions, including agents that target the noradrenergic, dopaminergic, serotonergic, cholinergic, glutamatergic, and GABAergic systems. Furthermore, several miscellaneous receptors have also been targeted, including neurokinin (NK), imidazoline, calcium channel, and endocannabinoid receptors, and genetic studies have also considered a number of putative anxiety-linked receptor targets, including the α_2 , NOP,

Table 1 Drugs effective in the MBT

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Drugs targeting the serotonergic system					
5-MeO-DMT	5-HT _{1A} & 5-HT ₂ agonist	0.25, 1, 2.5, 5 mg/kg i.p. 20 min pre	2.5, 5	–	(Njung'e & Handley, 1991a)
8-OH-DPAT	5-HT _{1A} agonist	0.3, 1, 3, 10 mg/kg i.p. 10 min pre	10	–	(Njung'e & Handley, 1991a)
		1, 3, 10 mg/kg i.p. 30 min pre	3, 10	#	(Ichimaru et al., 1995)
		0.1, 1, 3 mg/kg 30 min pre	3	/	(Matsushita et al., 2005)
		0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	0.63, 2.5	#	(Bruins Slot et al., 2008)
		3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
Buspirone	5-HT _{1A} agonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	20	–	(Njung'e & Handley, 1991a)
		15, 30, 60 mg/kg p.o. 60 min pre	30, 60	/	(Ichimaru et al., 1995)
		10, 20, 40, 80, 160 mg/kg p.o. 60 min pre	80, 160	–	(Abe et al., 1998)
		1, 3, 10, 30 mg/kg i.p. 30 min pre	3, 10, 30	–	(Nicolas et al., 2006)
Citalopram	Selective serotonin reuptake inhibitor	1, 3, 10, 30 mg/kg i.p. 30 min pre	10, 30	–	(Young et al., 2006)
		1, 5, 10, 20 mg/kg i.p. 30 min pre	20	/	(Njung'e & Handley, 1991a)
		5, 10, 15 mg/kg i.p. 20 min pre	5, 10, 15	/	(Takeuchi et al., 2002)
		1–3 mg/kg i.p. 30 min pre	1–3	#	(Li et al., 2006)
		0.3, 1, 3, 10, 30 mg/kg i.p. 30 min pre	3, 10, 30	–	(Nicolas et al., 2006)
Clomipramine	Serotonin–noradrenalin reuptake inhibitor	2.5, 10, 40 mg/kg s.c. 60 min pre	2.5–40	/	(Bruins Slot et al., 2008)
		10 mg/kg i.p. 30 min pre	10	/	(Krass et al., 2010)
		15, 30, 60 mg/kg p.o. 60 min pre	60	#	(Ichimaru et al., 1995)
		0.16, 0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	10, 40	–	(Millan et al., 2002)
		3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
DOI	5-HT _{2A/C} agonist	3, 10, 30 mg/kg i.p. 30 min pre	10, 30	–	(Nicolas et al., 2006)
		0.01, 0.02, 0.05, 0.1, 0.5, 1, 2.5, 5 mg/kg i.p. 30 min pre	0.1–5	/	(Njung'e & Handley, 1991a)
		0.3, 1 mg/kg i.p. 30 min pre	1	/	(Egashira, Okuno, et al., 2012)
Duloxetine	Serotonin–noradrenalin reuptake inhibitor	1, 3, 10, 30 mg/kg i.p. 30 min pre	10, 30	–	(Nicolas et al., 2006)
Escitalopram	Selective serotonin reuptake inhibitor	2 mg/kg s.c. 11 days	2	/	(Taylor et al., 2017)
Fenfluramine	5-HT releaser	1, 2.5, 5, 10 mg/kg i.p. 30 min pre	2.5, 5, 10	/	(Njung'e & Handley, 1991a)
Fluoxetine	Selective serotonin reuptake inhibitor	5, 10, 20, 40, 80, 160, 320 mg/kg p.o. 60 min pre	40, 80, 160	–	(Abe et al., 1998)
		5, 10, 15 mg/kg i.p. 20 min pre	15	/	(Takeuchi et al., 2002)
		3–30 mg/kg s.c. 30 min pre	30	/	(Li et al., 2006)
		3, 10, 30 mg/kg i.p. 30 min pre	10, 30	–	(Nicolas et al., 2006)
		5, 10, 15 mg/kg i.p. 30 min pre (acute)	10–15	/	(Uday et al., 2007)
		0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	2.5, 10	#	(Bruins Slot et al., 2008)
		5, 10, 15, 30 mg/kg s.c. 30 min pre (acute)	15, 30	/	(Umathe et al., 2008)
		2.5, 5 mg/kg s.c. twice daily 7 days (chronic)	5	/	(Umathe et al., 2008)
		1.5, 5, 10 mg/kg i.p. 30 min pre	10	/	(Umathe et al., 2009)
		10 mg/kg p.o. 60 min pre	10	#	(Badgujar & Surana, 2010)
		10 mg/kg i.p. 30 min pre	10	#	(Prajapati et al., 2011)
		2.5, 5, 10 mg/kg i.p. 30 min pre	5, 10	/	(Umathe et al., 2012)
		1, 3, 10 mg/kg i.p. 30 min pre	10	#	(Nardo et al., 2014)
Fluvoxamine	Selective serotonin reuptake inhibitor	5 mg/kg i.p. 30 min pre	5	#	(Kalariya et al., 2015)
		1, 5, 10, 20 mg/kg i.p. 30 min pre	10, 20	/	(Njung'e & Handley, 1991a)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
		15, 30, 60 mg/kg p.o. 60 min pre	30, 60	/	(Ichimaru et al., 1995)
		15, 30, 60 mg/kg/day p.o.	60	–	(Ichimaru et al., 1995)
		0.16, 0.63, 2.5, 10, 40 mg/kg s.c. 30 min pre	2.5, 10, 40	+	(Millan et al., 2002)
		3, 10, 30 mg/kg s.c. 30 min pre	10, 30	/	(Shimazaki et al., 2004)
		10, 30, 60 mg/kg i.p. 30 min pre	30, 60	/	(Harasawa et al., 2006)
		30 mg/kg p.o. 60 min pre	30	/	(Egashira, Harada, et al., 2007)
		2.5, 5, 10 mg/kg i.p. 4 h pre	5, 10	–	(Schneider & Popik, 2007)
		1, 5, 10 mg/kg i.p. 30 min pre	5, 10	/	(Sugimoto et al., 2007)
		30 mg/kg i.p. 30 min pre	30	/	(Matsushima et al., 2009)
		10, 30 mg/kg p.o. 60 min pre	30	/	(Egashira, Shirakawa, et al., 2012)
		30 mg/kg p.o. 60 min pre	30	/	(Egashira, Okuno, et al., 2012)
Gepirone	5-HT _{1A} agonist	5, 10, 20 mg/kg i.p. 30 min pre	10, 20	–	(Njung'e & Handley, 1991a)
Imipramine	Tricyclic antidepressant	4.6, 10, 22 mg/kg s.c. 30 min pre	10, 22	#	(Broekkamp et al., 1986)
		5–30 mg/kg i.p. 30 min pre	15–30	#	(Li et al., 2006)
Indalpine	Selective serotonin reuptake inhibitor	1, 5, 10, 20 mg/kg i.p. 30 min pre	5, 20	/	(Njung'e & Handley, 1991a)
Ketanserin	5-HT ₂ antagonist	1 mg/kg i.p. 30 min pre	1	–	(Njung'e & Handley, 1991a)
mCPP	Non-specific 5-HT agonist	1, 2.5, 5, 10, 20 mg/kg i.p. 30 min pre	2.5, 5, 10, 20	/	(Njung'e & Handley, 1991a)
		0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	–	(Nicolas et al., 2006)
		0.1, 0.3, 1 mg/kg i.p. 30 min pre	1	/	(Nardo et al., 2014)
MDMA	5-HT reuptake inhibitor/releaser	2.2, 4.4, 11, 22 µmol/kg i.p. 30 min pre	4.4–22	/	(Saadat et al., 2006)
Methysergide	5-HT _{1A} agonist; 5-HT ₂ antagonist	1, 5 mg/kg i.p. 30 min pre	5	–	(Njung'e & Handley, 1991a)
Mirtazepine	5-HT _{2/3} antagonist	1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Shirakawa, et al., 2012)
MKC-242	5-HT _{1A} agonist	0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 mg/kg p.o. 60 min pre	3.2, 6.4	–	(Abe et al., 1998)
Paroxetine	Selective serotonin reuptake inhibitor	0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	0.3, 1, 3	–	(Nicolas et al., 2006)
		2.6, 6.7, 13.6 µmol/kg i.p. 30 min pre	13.5	/	(Saadat et al., 2006)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira, Harada, et al., 2007)
		10 mg/kg i.p.	10	+	(Casarotto et al., 2010)
		10 mg/kg i.p. 30 min pre	10	/	(Krass et al., 2010)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira, Okuno, et al., 2012)
		3 mg/kg p.o. 60 min pre	3	/	(Egashira et al., 2018)
<i>Psilocybe argentipes</i> (raw plant material)	Non-specific 5-HT agonist	0.05, 0.1, 0.5, 1, 1.5, 2 g/kg p.o. 30 min pre	0.1, 0.5, 1	/	(Matsushima et al., 2009)
Psilocybin	Non-specific 5-HT agonist	0.025, 0.125, 0.25, 0.5, 1, 1.5 mg/kg p.o. 30 min pre	1.5	/	(Matsushima et al., 2009)
Ritanserin	5-HT _{2A/C} antagonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	20	–	(Njung'e & Handley, 1991a)
		0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	0.63, 2.5	#	(Bruins Slot et al., 2008)
RU-24,969	5-HT _{1A/B} agonist	0.1, 1, 2.5, 5, 10 mg/kg i.p. 30 min pre	2.5, 5, 10	+	(Njung'e & Handley, 1991a)
Tandospirone	5-HT _{1A} agonist	20, 40, 80, 160, 320 mg/kg p.o. 60 min pre	80, 160, 320	–	(Abe et al., 1998)
TFMPP	Non-specific 5-HT agonist	1, 5, 10, 20 mg/kg i.p. 30 min pre	5, 10, 20	–	(Njung'e & Handley, 1991a)
WAY-161503	5-HT _{2C} agonist	1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, et al., 2012)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
YM992	SSRI/5-HT _{2A} antagonist	5, 10, 15 mg/kg i.p. 20 min pre	15	/	(Takeuchi et al., 2002)
Zimeldine	Selective serotonin reuptake inhibitor	10 mg/kg i.p. 30 min pre	10	/	(Njung'e & Handley, 1991b)
		1, 3, 10, 15, 30 mg/kg i.p. 30 min pre	10, 15, 30	/	(Njung'e & Handley, 1991a)
		10 mg/kg p.o. 21 days	10	#	(Njung'e & Handley, 1991a)
Drugs targeting the dopaminergic system					
Amantidine	Dopamine reuptake inhibitor and releaser	3, 10, 30 mg/kg, i.p. 30 min pre test	30	/	(Egashira, Okuno, Harada, et al., 2008)
Amisulpride	D _{2/3} antagonist	0.63, 2.5, 10, 40 mg/kg s.c. 60 min pre	10, 40	#	(Bruins Slot et al., 2008)
Apomorphine	D ₂ agonist	0.046, 0.1, 0.22, 0.46 mg/kg s.c. 10 min pre	0.22, 0.46	#	(Broekkamp et al., 1986)
Aripiprazole	D ₂ antagonist/partial agonist; 5-HT _{1A} partial agonist	0.04, 0.16, 0.63, 2.5, 10 mg/kg i.p. 60 min pre	10	#	(Bruins Slot et al., 2008)
		0.3, 1 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Matsushita, et al., 2008)
Aripiprazole + Ethanol	D ₂ antagonist/partial agonist; 5-HT _{1A} partial agonist	0.1, 0.5, 4, 6 i.p. 30 min pre	0.5, 4, 6	/	(Gaikwad & Parle, 2011)
		0.1 mg/kg i.p. and 0.1% w/v i.p. (sub-effective doses)	0.1 + 0.1	/	(Gaikwad & Parle, 2011)
Bifeprunox	D ₂ ;5-HT _{1A} partial agonist	0.001, 0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg i.p. 60 min pre	0.001–0.01, 0.16–2.5	/	(Bruins Slot et al., 2008)
Bupropion	Dopamine reuptake inhibitor and releaser	20 mg/kg s.c. 30 min pre	20	+	(Honda et al., 2011)
Carbidopa	DOPA decarboxylase inhibitor	9 mg/kg i.p. 30 min pre	9	/	(Njung'e & Handley, 1991a)
Carbidopa + 5-Hydroxytryptophan	DOPA decarboxylase inhibitor + Serotonin precursor	9 mg/kg + 5, 10, 20 mg/kg i.p. 30 min pre	9 + 5, 10, 20	–	(Njung'e & Handley, 1991a)
Clozapine	Dopamine antagonist/ 5-HT ₂ antagonist	0.46, 1, 2.2, 4.6 mg/kg s.c. 30 min pre	2.2, 4.6	#	(Broekkamp et al., 1986)
		0.04, 0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	0.16–10	/	(Bruins Slot et al., 2008)
GBR-12909	DA reuptake inhibitor	1.9, 4.8, 9.7, 19.4 µmol/kg i.p. 30 min pre	9.7, 19.4	/	(Saadat et al., 2006)
Haloperidol	D ₂ antagonist	0.046, 0.1, 0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.46, 1	#	(Broekkamp et al., 1986)
		0.01, 0.03, 0.1 mg/kg i.p. 30 min pre	0.1	–	(Matsushita et al., 2005)
		0.03, 0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.3, 1	–	(Nicolas et al., 2006)
		0.0025, 0.01, 0.04, 0.16, 0.63 mg/kg s.c. 60 min pre	0.0025, 0.04–0.63	/	(Bruins Slot et al., 2008)
L-741,626	D ₂ antagonist	3, 10 mg/kg s.c. 30 min pre	10	–	(Egashira, Okuno, Matsushita, et al., 2008)
Olanzapine	D ₁₋₅ antagonist	0.3, 1, 3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
		0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(Bruins Slot et al., 2008)
PD-168,077	D ₄ agonist	2.5, 10, 40 mg/kg s.c. 60 min pre	40	#	(Bruins Slot et al., 2008)
Perospirone	D ₂ antagonist	3, 10 mg/kg p.o. 60 min pre	10	/	(Matsushita et al., 2005)
Perphenazine	D ₂ /D ₃ agonist	0.1, 0.22, 0.46, 1, 2.2 mg/kg s.c. 30 min pre	1, 2.2	#	(Broekkamp et al., 1986)
Pramipexole	D ₂ /D ₃ agonist	0.05, 0.5 mg/kg i.p.	0.5	–	(Jimenez-Gomez et al., 2011)
Quetiapine	D ₂ antagonist	10, 30, 100 mg/kg p.o. 60 min pre	100	–	(Egashira, Okuno, Matsushita, et al., 2008)
Quinpirole	D ₂ agonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	1	–	(Egashira, Okuno, Matsushita, et al., 2008)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Remoxipride	D _{2/3} antagonist	0.04, 0.16, 0.63, 2.5, 10, 40 mg/kg s.c. 60 min pre	40	#	(Bruins Slot et al., 2008)
Risperidone	D ₂ antagonist	0.1, 0.3, 1 mg/kg p.o. 60 min pre	1	–	(Matsushita et al., 2005)
		0.03–1 mg/kg i.p. 30 min pre	1	#	(Li et al., 2006)
		0.01, 0.04, 0.16, 0.63 mg/kg s.c. 60 min pre	0.16, 0.63	–	(Bruins Slot et al., 2008)
SLV-313	D ₂ antagonist/HT _{1A} agonist	1 mg/kg/day s.c. 21 days	1	#	(Torres-Lista et al., 2015)
		0.01, 0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(Bruins Slot et al., 2008)
Thiordazine	D ₂ /D ₃ antagonist	4.6, 10, 14, 46 mg/kg s.c. 30 min pre	10–46	#	(Broekkamp et al., 1986)
Trifluoperidol	D ₂ antagonist	0.1, 0.32, 1 mg/kg s.c. 30 min pre	0.32, 1	#	(Broekkamp et al., 1986)
Trifluoperazine	D ₁ /D ₂ antagonist	0.3, 1, 3 mg/kg p.o. 30 min pre	3	/	(Egashira et al., 2018)
Drugs targeting the adrenergic system					
Clonidine	α ₂ agonist; imidazoline I ₁ agonist	0.01, 0.03, 0.3 mg/kg i.p. 30 min pre	0.03, 0.3	–	(Young et al., 2006)
		15, 30, 60 mg/kg i.p. 30 min pre	60	#	(Dixit et al., 2014)
d-Amphetamine	Monoamine releaser/reuptake inhibitor*	0.1–10 mg/kg i.p. 10 min pre	1–10	+	(Li et al., 2006)
	Also affects DA, 5-HT	0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	+	(Nicolas et al., 2006)
		0.5, 1, 5 mg/kg i.p.	1, 5	+	(Jimenez-Gomez et al., 2011)
Desipramine	Noradrenalin reuptake inhibitor	3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
		10 mg/kg i.p. 4 h pre	10	–	(Schneider & Popik, 2007)
Methamphetamine	Monoamine releaser/reuptake inhibitor*	0.5, 2.7, 4.8 μmol/kg i.p. 30 min pre	2.7, 4.8	/	(Saadat et al., 2006)
	Also affects DA, 5-HT				
Methylphenidate	Monoamine releaser/reuptake inhibitor*	9.3, 18.5, 37 μmol/kg i.p. 30 min pre	37	/	(Saadat et al., 2006)
	Also affects DA, 5-HT				
Mianserin	Noradrenalin reuptake inhibitor; anti-serotonergic	3.2, 10, 32 mg/kg s.c. 30 min pre	10, 32	#	(Broekkamp et al., 1986)
Milnacipran	Serotonin-noradrenalin reuptake inhibitor	5, 10, 20 mg/kg i.p. 30 min pre	10, 20	/	(Sugimoto et al., 2007)
		20 mg/kg s.c. 30 min pre	20	/	(Honda et al., 2011)
Nisoxetine	Noradrenalin reuptake inhibitor	3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
Nomifensine	Noradrenalin reuptake inhibitor	10 mg/kg i.p. 4 h pre	10	+	(Schneider & Popik, 2007)
		0.0025, 0.01, 0.04, 0.16, 0.63, 2.5 mg/kg	2.5	#	(Bruins Slot et al., 2008)
para--chloroamphetamine (PCA)	Monoamine releaser/reuptake inhibitor*	15 mg/kg 2 doses 6 hours apart (neurotoxic dose, day 28 post)	15	#	(Saadat et al., 2006)
	Also affects DA, 5-HT	2.4, 3.9, 4.8, 9.7 μmol/kg i.p. 30 min pre	4.8–9.7	/	(Saadat et al., 2006)
Prazosin	α ₁ antagonist	0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	2.5	#	(Bruins Slot et al., 2008)
TDIQ	α ₂ partial agonist	0.03, 0.3, 1, 3, 10, 17 mg/kg i.p. 30 min pre	1, 3, 10, 17	/	(Young et al., 2006)
Venlafaxine	Serotonin-noradrenalin reuptake inhibitor	10–60 mg/kg i.p. 30 min pre	30–60	#	(Li et al., 2006)
Yohimbine	α ₂ antagonist; anxiogenic	1, 5, 10 mg/kg i.p. 30 min pre	5, 10	–	(Njung'e & Handley, 1991b)
		0.3, 1, 3, 10 mg/kg i.p. 30 min pre	3, 10	–	(Nicolas et al., 2006)
Drugs targeting the GABAergic system					
(+)-limonene epoxide	GABA potentiator	25, 50, 75 mg/kg p.o. 30 min pre	25, 50, 75	#	(De Almeida et al., 2014)
		25, 50, 75 mg/kg/day p.o. 14 days	25, 50, 75	#	(De Almeida et al., 2014)
Allopregnanolone	GABA _A potentiator	0.1, 0.2, 0.5, 1 μg/kg i.c.v. 15 min pre	1	/	(Umathe et al., 2009)
Alprazolam	Benzodiazepine receptor agonist	0.03, 0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.3, 1	–	(Nicolas et al., 2006)
Baclofen	GABA _B agonist	3, 10 mg/kg i.p. 30 min pre	10	–	(Egashira et al., 2013)
βCCM (anxiogenic)	Benzodiazepine receptor inverse agonist	0.1, 1 mg/kg i.p. 0 min pre	1	–	(Jimenez-Gomez et al., 2011)
Chlordiazepoxide	Benzodiazepine receptor agonist	4.6, 10, 22, 46 mg/kg s.c. 30 min pre	10, 22	#	(Broekkamp et al., 1986)
		3–30 mg/kg i.p. 30 min pre	30	#	(Li et al., 2006)
		10, 30, 100 mg/kg i.p. 30 min pre	30, 100	–	(Nicolas et al., 2006)
Clonazepam	Benzodiazepine receptor agonist	0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.22–1	#	(Broekkamp et al., 1986)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Diazepam	Benzodiazepine receptor agonist	0.46, 1, 2.2, 4.6, 10 mg/kg s.c. 30 min pre	2.2, 4.6, 10	#	(Broekkamp et al., 1986)
		0.05, 0.1, 0.25, 1, 5 mg/kg i.p. 30 min pre	1, 5	+	(Njung'e & Handley, 1991b)
		1, 3, 10 mg/kg p.o. 60 min pre	10	/	(Ichimaru et al., 1995)
		0.25–32 mg/kg p.o. 60 min pre	4, 8	–	(Abe et al., 1998)
		1, 3, 10 mg/kg i.p. 30 min pre	3, 10	/	(Nicolas et al., 2006)
		0.03, 0.3, 1, 3, 10, 30 mg/kg i.p. 30 min pre	3, 10, 30	–	(Young et al., 2006)
		2 mg/kg i.p. 1 h pre	2	–	(Schneider & Popik, 2007)
		0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	2.5, 10	+	(Bruins Slot et al., 2008)
		2.5 mg/kg i.p. 30 min pre	2.5	/	(Casarotto et al., 2010)
		0.5, 1, 5 mg/kg i.p.	1, 5	/	(Jimenez-Gomez et al., 2011)
		0.3, 1, 3 mg/kg i.p. 60 min pre	1, 3	–	(Kinsey et al., 2011)
		0.5, 2 mg/kg i.p. 30 min pre	2	?	(Umathe et al., 2012)
		2 mg/kg p.o. 30 min pre	2	#	(De Almeida et al., 2014)
		2 mg/kg/day p.o. 14 days	2	#	(De Almeida et al., 2014)
		2 mg/kg s.c. 11 days	2	/	(Taylor et al., 2017)
Ethanol	GABA potentiator; NMDA receptor antagonist	460, 1000, 2200, 3200 mg/kg s.c. 30 min pre	1,100–3,200	#	(Broekkamp et al., 1986)
		Withdrawal from addiction schedule	–	–	(Umathe et al., 2008)
		0.1, 1.2, 2.4, 4.8% w/v solution i.p. 30 min pre	1.2, 2.4, 4.8	/	(Gaikwad & Parle, 2011)
Flunitrazepam	Benzodiazepine receptor agonist	0.046, 0.1, 0.46 mg/kg s.c. 30 min pre	0.1, 0.46	#	(Broekkamp et al., 1986)
Meprobamate	GABA receptor potentiator	22, 46, 100, 150, 220 mg/kg s.c. 30 min pre	150, 220	#	(Broekkamp et al., 1986)
Muscimol	GABA _A agonist	0.3, 1 mg/kg i.p. 40 min pre	1	/	(Egashira et al., 2013)
Tiagabine	GABA reuptake inhibitor	1, 3, 10 mg/kg i.p. 30 min pre	3, 10	–	(Nicolas et al., 2006)
Drugs targeting the glutamatergic system					
CX-546	AMPA receptor potentiator	3, 10, 30 mg/kg s.c. 30 min pre	30	/	(Iijima et al., 2010)
Fenobam	mGluR ₅ antagonist	30, 100, 300 mg/kg i.p. 30 min pre	300	+	(Nicolas et al., 2006)
LY341495	mGluR ₂ antagonist	1, 3, 10 mg/kg i.p. 60 min pre	1, 3, 10	#	(Shimazaki et al., 2004)
Memantine	NMDA antagonist	1, 3, 10 mg/kg, i.p. 30 min pre test	10	/	(Egashira, Okuno, Harada, et al., 2008)
MGS0039	mGluR _{2/3} antagonist	1, 3, 10 mg/kg i.p. 60 min pre	3, 10	/	(Shimazaki et al., 2004)
MK-801	NMDA antagonist	0.03, 0.1, 0.3 mg/kg i.p. 30 min pre test	0.3	+	(Egashira, Okuno, Harada, et al., 2008)
MTEP	mGluR ₅ antagonist	1, 3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Nicolas et al., 2006)
Ro 25-6981	NMDA antagonist	0.3, 1, 3 mg/kg s.c. 30 min pre	3	/	(Iijima et al., 2010)
Drugs targeting the endocannabinoid system					
AM404	Endocannabinoid reuptake inhibitor	0.3, 1, 3 mg/kg i.p. 30 min pre	0.3, 1, 3	/	(Gomes et al., 2011)
		0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	0.1, 1, 5	/	(Umathe et al., 2012)
Anandamide	Endocannabinoid CB ₁ agonist	0.1, 0.5, 1, 5, 10, 20, 40 µg/mouse i.c.v. 2 min pre	1, 5, 10	/	(Umathe et al., 2012)
Anandamide + AM404	Endocannabinoid CB ₁ agonist	0.5 µg/mouse + 0.05 µg/mouse i.c.v. (sub-effective doses)	0.5 + 0.05	/	(Umathe et al., 2012)
Anandamide + URB597	Endocannabinoid CB ₁ agonist	0.5 µg/mouse + 0.01 µg/mouse i.c.v. (sub-effective doses)	0.5 + 0.01	/	(Umathe et al., 2012)
Cannabidiol	CB ₁ antagonist	5, 15, 30, 60 mg/kg i.p. 30 min pre	15, 30, 60	/	(Casarotto et al., 2010)
		30 mg/kg/day 7 days (chronic)	30	#	(Casarotto et al., 2010)
		5, 15, 30 mg/kg i.p. 30 min pre	30	#	(Nardo et al., 2014)
Cannabidiol + Fluoxetine	CB ₁ antagonist + SSRI	3 mg/kg + 15 mg/kg i.p. 30 min pre (sub-effective doses)	3 + 15	#	(Nardo et al., 2014)
Capsazepine	TRPV1 antagonist	0.1, 1, 10, 100 µg/mouse i.c.v. 2 min pre	100	/	(Umathe et al., 2012)
JZL184	MAGL inhibitor; cannabinoid potentiator	4, 16, 40 mg/kg i.p. 2 h pre	16, 40	–	(Kinsey et al., 2011)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
PF-3845	FAAH Inhibitor; cannabinoid potentiator	1, 3, 10 mg/kg i.p. 2h pre	10	/	(Kinsey et al., 2011)
Tetrahydrocannabinol (THC)	Cannabinoid CB ₁ agonist	0.03, 0.1, 0.3, 1, 3, 10, 30 mg/kg i.p. 60 min pre	0.3, 10, 30	–	(Kinsey et al., 2011)
URB597	FAAH inhibitor; cannabinoid potentiator	0.1, 0.3, 1 mg/kg i.p. 30 min pre 0.01, 0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	0.1, 0.3, 1 0.05–5	/	(Gomes et al., 2011) (Umathe et al., 2012)
WIN 55,212-2	Cannabinoid CB ₁ agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	–	(Gomes et al., 2011)
Drugs targeting miscellaneous targets					
(+)-SKF 10047	Sigma σ_1 agonist	3, 10mg/kg i.p. 30 min pre	10	/	(Egashira, Harada, et al., 2007)
2-BFI	Imidazoline I ₂ agonist	5, 10, 15 mg/kg i.p. 30 min pre	15	#	(Dixit et al., 2014)
7-Nitroindazole (7-NI)	Nitric oxide synthase inhibitor	20, 50 mg/kg i.p. 30 min pre 10, 20, 40 mg/kg i.p. 30 min pre	50 20, 40	– /	(Krass et al., 2010) (Gawali et al., 2016)
Agmatine	Imidazoline receptor agonist; Nitric oxide synthesis inhibitor	10, 50 mg/kg 30 i.p. min pre 10, 20, 40 mg/kg i.p. 30 min pre 10, 20, 40 mg/kg i.p. 30 min pre	10, 50 20, 40 20, 40	/	(Krass et al., 2010) (Dixit et al., 2014) (Gawali et al., 2016)
Agmatine + clonidine	Imidazoline I ₁ /α ₂ agonist	10 mg/kg + 30 mg/kg (subeffective doses)	10 + 30	#	(Dixit et al., 2014)
Agmatine + moxonidine	Imidazoline I ₁ /α ₂ agonist	10 mg/kg + 0.25 mg/kg (subeffective doses)	10 + 0.25	#	(Dixit et al., 2014)
Agmatine + 2-BFI	Imidazoline I ₂ agonist	10 mg/kg + 10 mg/kg (subeffective doses)	10 + 10	#	(Dixit et al., 2014)
Agmatine + 7-NI	Nitric oxide synthesis inhibitor	10 mg/kg + 10 mg/kg (subeffective doses)	10 + 10	/	(Gawali et al., 2016)
Amlodipine	Calcium channel blocker	3, 10 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Abe, et al., 2008)
Ascorbic acid	Dietary supplement	250 mg/kg p.o. 30 min pre 250 mg/kg/day p.o. 14 days	250 250	#	(De Almeida et al., 2014) (De Almeida et al., 2014)
Atropine	Anticholinergic	0.46, 1, 2.2, 4.6, 10 mg/kg s.c. 30 min pre 1, 3, 10 mg/kg i.p. 30 min pre	1–4.6 10	#	(Broekkamp et al., 1986) (Nicolas et al., 2006)
Carbamazepine	Anti-Epileptic	10, 100, 300 mg/kg p.o. 60 min pre	200, 300	–	(Egashira et al., 2013)
Chlorpromazine	Antihistamine	0.32, 1, 3.2 mg/kg s.c. 30 min pre 1.25, 2.5, 5, 10 mg/kg p.o. 60 min pre 1–10 mg/kg i.p. 30 min pre	1, 3.2 10 10	#	(Broekkamp et al., 1986) (Abe et al., 1998) (Li et al., 2006)
Chlorpyrifos (Organophosphate)	Acetylcholine esterase inhibitor	0.3, 1, 3 mg/kg i.p. 30 min pre 1 mg/kg/day i.p. 5 days	3 1	–	(Nicolas et al., 2006) (Savy et al., 2015)
Cilnidipine	Calcium channel blocker	3, 10 mg/kg i.p. 30 min pre	10	/	(Egashira, Okuno, Abe, et al., 2008)
Cyproheptadine	Antihistamine	1, 2, 5 mg/kg i.p. 30 min pre	5	–	(Njung'e & Handley, 1991a)
Diazinon	Acetylcholine esterase inhibitor	1 mg/kg/day i.p. 5 days	1	/	(Savy et al., 2015)
Diphenhydramine	Acetylcholine esterase inhibitor	2.2, 6.8, 22 mg/kg s.c. 30 min pre	22	#	(Broekkamp et al., 1986)
<i>Dolichandrone falcata</i> ethyl acetate extract	Novel plant extract	100, 200, 400 mg/kg p.o. 60 min pre	200, 400	#	(Badgujar & Surana, 2010)
<i>Dolichandrone falcata</i> TLC isolated compounds	Novel plant extract	50, 100, 200 mg/kg p.o. 60 min pre	50, 100, 200	#	(Badgujar & Surana, 2010)
Flunarizine	Calcium channel blocker	3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Egashira, Okuno, Abe, et al., 2008)
GR205,171	Neurokinin NK ₁ receptor antagonist	0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	10, 40	–	(Millan et al., 2002)
HECE (<i>C. esculenta</i> extract)	Novel plant extract	25, 50 mg/kg i.p. 30 min pre	25, 50	#	(Kalariya et al., 2015)
<i>Hypericum perforatum</i> extract	Novel plant extract	150, 300 mg/kg p.o. 60 min pre	150, 300	/	(Skalisz et al., 2004)
Lamotrigine (ddY mice)	Anti-epileptic; sodium channel blocker	3, 10, 30 mg/kg i.p. 30 min pre	30	/	(Egashira et al., 2013)
<i>Lagenaria siceraria</i> extract	Novel plant extract	25, 50 mg/kg i.p. 30 min pre	25, 50	#	(Prajapati et al., 2011)
L-NAME	Neuronal nitric oxide synthase inhibitor	15, 30, 50 mg/kg 30 min pre	30, 50	/	(Gawali et al., 2016)

Table 1 (continued)

Drug	Mechanism of action	Dose range	ED	LMA	Reference
L-NAME + Agmatine	Neuronal nitric oxide synthase inhibitor	15 mg/kg + 10 mg/kg (sub-effective doses)	15 + 10	/	(Gawali et al., 2016)
Leuprolide	Gonadotropin releasing hormone analogue	50, 100, 300, 600 µg/kg s.c. 30 min pre (acute)	100–600	/	(Uday et al., 2007)
		50, 100, 300, 600 µg/kg s.c. 30 min pre (acute)	100–600	/	(Umathe et al., 2008)
		25, 50 µg/kg s.c. twice daily 7 days (chronic)	50	/	(Umathe et al., 2008)
		100, 200, 300 µg/kg s.c. 30 min pre	200, 300	/	(Gaikwad et al., 2010)
Leuprolide + Fluoxetine	Gonadotropin releasing hormone analogue + SSRI	50 µg/kg s.c. & 5 mg/kg i.p. 30 min pre (sub-effective doses)	50 + 5	/	(Uday et al., 2007)
MCH (Melanin concentrating hormone)	Diverse hormone	i.c.v. 2 min pre	-	/	(Sanathara et al., 2018)
Metergoline	Dopamine agonist	0.1, 0.25, 1	1	–	(Njung'e & Handley, 1991a)
Morphine	Opioid analgesic	0.3, 1, 3, 10 mg/kg i.p. 30 min pre	10	+	(Nicolas et al., 2006)
Moxonidine	Imidazoline I ₂ receptor agonist	0.25, 0.5, 1 mg/kg i.p. 30 min pre	0.5, 1	#	(Dixit et al., 2014)
N-acetyl-L-cysteine (NAC)	Anti-oxidant; mucolytic	50, 100, 150 mg/kg i.p. 30 min pre	150	/	(Egashira, Shirakawa, et al., 2012)
Nilvadipine	Calcium channel blocker	1, 3, 10 mg/kg i.p. 30 min pre	3, 10	/	(Egashira, Okuno, Abe, et al., 2008)
Oxytocin	Hormone	i.c.v. 2 min pre	-	/	(Sanathara et al., 2018)
Phenelzine	Monoamine oxidase inhibitor	3, 10, 30, 100 mg/kg i.p. 30 min pre	100	/	(Nicolas et al., 2006)
Progesterone	Sex hormone	1 mg/kg s.c. 4h pre	1	–	(Schneider & Popik, 2007)
		4 mg/kg s.c. 3h pre	4	+	(Llaneza & Frye, 2009)
		5, 10, 20 mg/kg s.c. 60 min pre	20	/	(Umathe et al., 2009)
Progesterone + 17β-Estradiol	Sex hormone	4 mg/kg & 10 µg/rat	4 + 10	+	(Llaneza & Frye, 2009)
PRE-084	Sigma σ ₁ agonist	10, 30, 60 mg/kg i.p. 30 min pre	60	/	(Egashira, Harada, et al., 2007)
Ro64-6198	Nociceptin opioid peptide receptor agonist	0.1, 0.3, 1, 3 mg/kg i.p. 30 min pre	1, 3	–	(Nicolas et al., 2006)
RP67,580	Neurokinin NK ₁ receptor antagonist	0.63, 2.5, 10, 40 mg/kg s.c. 30 min pre	10, 40	–	(Millan et al., 2002)
Scopolamine	Anticholinergic	0.046, 0.1, 0.22, 0.46, 1 mg/kg s.c. 30 min pre	0.046–1	#	(Broekkamp et al., 1986)
Tipepidine	GIRK inhibitor	5, 10, 20 mg/kg s.c. 30 min pre	5, 10, 20	+	(Honda et al., 2011)
TRIM	Nitric oxide synthase inhibitor	25, 50 mg/kg i.p. 30 min pre	25, 50	/	(Krass et al., 2010)
Valproate	Anti-epileptic; GABA potentiator	10, 30, 100 mg/kg i.p. 30 min pre	10, 30, 100	/	(Egashira et al., 2013)
<i>Withania somnifera</i> extract	Novel plant extract	10, 20, 40 mg/kg p.o. 12 days	20, 40	#	(Dey et al., 2016)

ED: effective dose; LMA: locomotor activity (/ no effect; # not measured/reported; + increased; – decreased)

NP-Y, TPH2, VDR, and V1a receptors (Angoa-Pérez et al., 2013; Egashira, Tanoue, et al., 2007; Gavioli et al., 2007; Lähdesmäki et al., 2002; Tasan et al., 2009). Taking this into account, it therefore must be considered that if marble-burying activity is triggered and driven by a possible anxiogenic and/or OC-like construct, the test itself as applied in the preclinical literature has shown poor predictive validity as a screening test for either behavior. Indeed, neither clinical anxiety nor OCD has demonstrated a response to many of the pharmacological agents listed in Table 1. Furthermore, even if it can be argued that preclinical experimental investigations may suffice only to contribute to our understanding of disease mechanisms, and that the answers obtained from such investigations do not have to

contribute to clinical treatment strategies, the findings reported in Tables 1, 2, and 3 are, in retrospect, also of little translational value. In this section, we will address this dilemma from a translational perspective.

Serotonergic drugs in studies of marble-burying behavior

Serotonin reuptake inhibitors Drugs targeting the serotonergic systems have, given their widespread application in OCD, anxiety, and other stress-related disorders (Bandelow et al., 2012; Bandelow et al., 2008; Dougherty et al., 2004), been

Table 2 Drugs exacerbating MBA/Blocking otherwise effective agents

Drug	Mechanism of action	Dose range	ED	LMA	Reference
Drugs targeting the serotonergic system					
ICI 169,369 PT to DOI	5-HT ₂ antagonist	10 mg/kg i.p. 40 min pre	10	/	(Njung'e & Handley, 1991a)
Ketanserin PT to trifluoperazine	5-HT ₂ antagonist	0.3 mg/kg i.p.	0.3	/	(Egashira et al., 2018)
mCPP	Non-specific 5-HT agonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	0.1, 0.3	/	(Nardo et al., 2014)
NAN-190 PT to fluvoxamine	5-HT _{1A} antagonist	0.3 mg/kg s.c. 30 min pre	0.3	#	(Ichimaru et al., 1995)
PCPA PT to leuprolide; fluoxetine	5-HT synthesis inhibitor	300 µg/kg i.p. 3 days	300	/	(Uday et al., 2007)
Ritanserin PT to leuprolide	5-HT _{2A/C} antagonist	20 mg/kg i.p. 30 min pre	20	/	(Gaikwad et al., 2010)
Ritanserin PT to DOI		0.2, 0.5, 1 mg/kg i.p. 40 min pre	0.5, 1	/	(Njung'e & Handley, 1991a)
SB-242084 PT to DOI	5-HT _{2C} antagonist	3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, et al., 2012)
SB-242084 PT to fluvoxamine; paroxetine		1 mg/kg i.p. 30 min pre	1	+	(Egashira, Okuno, et al., 2012)
SB-242084 PT to WAY-161503		3 mg/kg i.p. 30 min pre	3	+	(Egashira, Okuno, et al., 2012)
WAY-100135 PT to 8-OH-DPAT; perospirone	5-HT _{1A} antagonist	10 mg/kg i.p. 30 min	10	#	(Matsushita et al., 2005)
WAY-100635 PT to fluvoxamine	5-HT _{1A} antagonist	0.1, 1 mg/kg i.p. 60 min pre	1	/	(Harasawa et al., 2006)
WAY-100635 PT to 8-OH-DPAT		3 mg/kg i.p. 30 min pre	3	/	(Egashira, Okuno, Matsushita, et al., 2008)
WAY-100635 PT to paroxetine		3 mg/kg i.p. 30 min pre	3	#	(Casarotto et al., 2010)
WAY-100635 PT to tipecidine		3 mg/kg s.c. 60 min pre	3	+	(Honda et al., 2011)
Drugs targeting the dopaminergic system					
Raclopride PT to tipecidine	D ₂ antagonist	0.4 mg/kg s.c. 60 min pre	0.4	/	(Honda et al., 2011)
Quinpirole PT to trifluoperazine	D ₂ agonist	0.03 mg/kg i.p.	0.03	/	(Egashira et al., 2018)
Drugs targeting the adrenergic system					
Efaroxan PT to agmatine	α ₂ antagonist; imidazoline receptor antagonist	2 mg/kg i.p. 30 min pre	2	#	(Dixit et al., 2014)
Idazoxan PT to agmatine	α ₂ antagonist; imidazoline receptor antagonist	1 mg/kg i.p. 30 min pre	1	#	(Dixit et al., 2014)
Drugs targeting the GABAergic system					
Bicuculline PT to muscimol, valproate	GABA _A receptor antagonist	3 mg/kg 30 min pre	3	/	(Egashira et al., 2013)
Flumazenil PT to (+)-limonene epoxide; diazepam	Benzodiazepine receptor antagonist	5 mg/kg p.o. 60 min pre	5	#	(De Almeida et al., 2014)
Flumazenil PT to (+)-limonene epoxide; diazepam		5 mg/kg/day p.o. 14 days	5	#	(De Almeida et al., 2014)
Drugs targeting the glutamatergic system					
LY354740 PT to MGS0039	mGluR _{2/3} agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	3	#	(Shimazaki et al., 2004)
Drugs targeting the endocannabinoid system					
AM-251 PT to cannabidiol	CB ₁ antagonist	1 mg/kg i.p. 30 min pre	1	#	(Casarotto et al., 2010)
AM-251 PT to WIN 55,212-2; URB597		1 mg/kg i.p. 60 min pre	1	/	(Gomes et al., 2011)
AM-251 PT to AN, AM404; URB597		1 µg/mouse i.c.v. 2 min pre	1	/	(Umathe et al., 2012)
AM404	Endocannabinoid reuptake inhibitor	0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	20	/	(Umathe et al., 2012)
Anandamide	Endocannabinoid CB ₁ agonist	0.1, 0.5, 1, 5, 10, 20, 40 µg/mouse i.c.v. 2 min pre	40	/	(Umathe et al., 2012)
Capsaicin	TRPV1 agonist	0.1, 1, 10, 100 µg/mouse i.c.v. 2 min pre	100	/	(Umathe et al., 2012)
Rimonabant PT to PF-3845; JZL184	CB ₁ antagonist	0.3 mg/kg i.p. 10 min pre drug	0.3	/	(Kinsey et al., 2011)
URB597	FAAH inhibitor; cannabinoid potentiator	0.01, 0.05, 0.1, 1, 5, 10, 20 µg/mouse i.c.v. 2 min pre	20	/	(Umathe et al., 2012)
Drugs targeting miscellaneous systems					
BD 1047 PT to fluvoxamine	Sigma σ _{1/2} antagonist	1, 3, 10 mg/kg i.p. 30 min pre	3	/	(Egashira, Harada, et al., 2007)
BD 1063 PT to fluvoxamine	Selective sigma σ ₁ antagonist	0.3, 1 mg/kg 30 min pre	1	/	(Egashira, Harada, et al., 2007)
Cyproheptadine PT to DOI	Antihistamine	2 mg/kg i.p. 40 min pre	2	/	(Njung'e & Handley, 1991a)
DHAS (dehydroisoandrosterone 3-sulphate)	Neurosteroid	1, 2.5, 5 mg/kg i.p. 30 min pre	5	/	(Umathe et al., 2009)
GW803430 PT to oxytocin and MCH	Melanin concentrating hormone antagonist	3 mg/kg i.p. 30 min pre	3	/	(Sanathara et al., 2018)
L-arginine	Dietary supplement; nitric oxide precursor	200, 400, 800 mg/kg i.p. 30 min pre	800	/	(Gawali et al., 2016)
L-arginine 500 mg/kg PT to paroxetine; citalopram	Dietary supplement; nitric oxide precursor	500 mg/kg i.p. 10 min pre	500	#	(Krass et al., 2010)
LHRH antagonist, PT to leuprolide; fluoxetine	Sex hormone modulator	2.5 µg/mouse	2.5	/	(Uday et al., 2007)

ED: effective dose; LMA: locomotor activity; PT: pretreatment (/ no effect; # not measured/reported; + increased; – decreased)

Table 3 Drugs ineffective in the MBT

Drug	Mechanism of action	Dose range	LMA	Reference
Drugs targeting the serotonergic system				
Buspirone	5-HT _{1A} agonist	0.3–10 mg/kg i.p. 30 min pre	#	(Li et al., 2006)
		0.01–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
Desipramine	Noradrenalin reuptake inhibitor	15, 30, 60 mg/kg p.o. 60 min pre	#	(Ichimaru et al., 1995)
		1, 3, 10, 30 mg/kg i.p. 30 min pre	/	(Nicolas et al., 2006)
Escitalopram	Selective serotonin reuptake inhibitor	50 mg/kg/day p.o. 28 days (chronic)	–	(Wolmarans et al., 2016)
ICI 169,369	5-HT ₂ antagonist	1, 5, 10 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
ICS 205-930	5-HT ₃ antagonist	0.1, 1, 10 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)
Ipsapirone	5-HT _{1A} partial agonist	5, 10, 20 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
Ketanserin	5-HT ₂ antagonist	3 mg/kg i.p. 30 min pre	/	(Egashira, Okuno, Matsushita, et al., 2008)
		0.3 mg/kg i.p. 60 min pre	/	(Egashira et al., 2018)
LY266097	5-HT _{2B} antagonist	0.16–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
MDL100907	5-HT _{2A} receptor antagonist	-	#	(Bruins Slot et al., 2008)
MDMA	5-HT reuptake inhibitor	25 mg/kg 3 doses 3 hourly i.p. (neurotoxic dose)	#	(Saadat et al., 2006)
Metergoline	5-HT _{1/2} antagonist	0.25 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991a)
Ondansetron	5-HT ₃ antagonist	0.01, 0.1, 1 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)
Ritanserin	5-HT _{2A/C} antagonist	1, 2, 20 mg/kg i.p. 30 min pre	/	(Gaikwad et al., 2010)
RS127445	5-HT _{2B} antagonist	0.0025–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SB-215505	5-HT _{2B} antagonist	0.16–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SB242084	5-HT _{2C} receptor antagonist	0.01–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
		1, 3 mg/kg i.p. 30 min pre	/	(Egashira, Okuno, et al., 2012)
S15535	5-HT _{1A} partial agonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
WAY-100635	5-HT _{1A} antagonist	1 mg/kg i.p. 60 min pre	/	(Harasawa et al., 2006)
		0.04–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
		3 mg/kg s.c. 60 min pre	/	(Honda et al., 2011)
Drugs targeting the dopaminergic system				
ABT-724	D ₄ agonist	0.63–40 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
BP897	D ₃ partial agonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
F-15063	D ₂ /D ₃ antagonist, D ₄ agonist; 5-HT _{1A} partial agonist	0.01–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
L745870	D ₄ antagonist	0.01–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Quinpirole	D _{2/3} agonist	0.03 mg/kg i.p. 60 min pre	/	(Egashira et al., 2018)
Raclopride	D ₂ antagonist	0.4 mg/kg s.c. 60 min pre	/	(Honda et al., 2011)
Ro10-5824	D ₄ partial agonist	2.5–40 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Sarizotan	5-HT _{1A} agonist; D ₂ antagonist	0.16, 0.63, 2.5, 10 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
SB-277,011-A	D ₃ antagonist	0.01–10 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
SCH23390	D ₁ antagonist	7.5 µg/mouse s.c. 60 min pre	/	(Honda et al., 2011)
SCH 23390 PT to tipepidine	D ₁ antagonist	7.5 µg/mouse s.c. 60 min pre	/	(Honda et al., 2011)
SSR181507	D ₂ antagonist/partial agonist; 5-HT _{1A} agonist	0.04, 0.16, 0.63, 2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
S33084	D ₃ antagonist	0.04–2.5 mg/kg i.p. 60 min pre	#	(Bruins Slot et al., 2008)
Drugs targeting the adrenergic system				
Atipamezole	α ₂ antagonist	0.04–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
Pindolol	Beta blocker; 5-HT _{1A} antagonist	5, 10 mg/kg i.p. 30 min pre	#	(Njung'e & Handley, 1991a)

Table 3 (continued)

Drug	Mechanism of action	Dose range	LMA	Reference
Drugs targeting the GABAergic system				
β CCE (β -carboline)	Benzodiazepine receptor inverse agonist	1, 5 mg/kg i.p. 30 min pre	/	(Njung'e & Handley, 1991b)
Bicuculline	GABA _A antagonist	3 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Bretazenil	GABA _A receptor modulator	1–100 mg/kg i.p. 30 min pre	#	(Li et al., 2006)
FG-7142 (β -carboline)	Benzodiazepine receptor inverse agonist	1, 3, 10, 30 mg/kg i.p. 30 min pre	+	(Nicolas et al., 2006)
Flumazenil	Benzodiazepine receptor antagonist	5 mg/kg p.o. 30 min pre	#	(De Almeida et al., 2014)
Pentobarbital	Barbiturate; GABA _A potentiator	3–17 mg/kg i.p. 15 min pre	#	(Li et al., 2006)
Drugs targeting the glutamatergic system				
LY354740	mGluR _{2/3} agonist	0.3, 1, 3 mg/kg i.p. 30 min pre	#	(Shimazaki et al., 2004)
NBQX	AMPA antagonist	0.1, 0.3, 1 mg/kg i.p. 30 min pre	–	(Egashira, Okuno, Harada, et al., 2008)
Riluzole	Glutamate release inhibitor	0.3, 1, 10 mg/kg i.p. 30 min pre	–	(Egashira, Okuno, Harada, et al., 2008)
Drugs targeting miscellaneous systems				
17 β -Estradiol	Sex hormone	10 μ g/rat 44–48 h pre	+	(Llaneza & Frye, 2009)
α -tocopherol	Dietary supplement	10, 30, 100 mg/kg p.o. 60 min pre	/	(Egashira, Shirakawa, et al., 2012)
Chlorpromazine	Antihistamine	2 mg/kg i.p. 1h pre	/	(Schneider & Popik, 2007)
Efaroxan	Imidazoline I ₁ /I ₂ antagonist	0.5, 1, 2 mg/kg i.p. 30 min pre	#	(Dixit et al., 2014)
Finasteride	5 α -reductase inhibitor	50 mg/kg i.p. 90 min pre	/	(Umathe et al., 2009)
GR226206	Neurokinin NK ₁ antagonist	0.63, 2.5, 10, 40 mg/kg i.p. 30 min pre	/	(Millan et al., 2002)
Hypericum perforatum extract	Novel plant extract	150, 300 mg/kg/day p.o. 21 days	/	(Skalisz et al., 2004)
Idazoxan	Imidazoline I ₁ /I ₂ antagonist	0.25, 0.5, 1 mg/kg i.p. 30 min pre	#	(Dixit et al., 2014)
L-arginine	Dietary supplement; Nitric oxide precursor	500 mg/kg i.p. 10 min pre	?	(Krass et al., 2010)
Lamotrigine (ICR mice)	Anti-epileptic	3, 10, 30 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Lithium (ICR mice)	Mood stabilizer	10, 30, 100 mg/kg i.p. 30 min pre	/	(Egashira et al., 2013)
Pregabalin	Anti-epileptic; calcium channel inhibitor	3, 10, 30, 100 mg/kg i.p. 30 min pre	–	(Nicolas et al., 2006)
Pyrilamine	Antihistamine	0.04–2.5 mg/kg s.c. 60 min pre	#	(Bruins Slot et al., 2008)
SM-21	Selective sigma σ_2 antagonist	0.3, 1, 10 mg/kg i.p. 30 min pre	+	(Egashira, Harada, et al., 2007)

LMA: locomotor activity (/ no effect; # not measured/reported; + increased; – decreased)

well represented in the MBT. SSRI treatment, acting via increased synaptic 5-HT concentrations (Goddard et al., 2008), is the first line choice for the treatment of both anxiety (Bandelow et al., 2012; Bandelow et al., 2008) and OCD (Abramowitz et al., 2009; Fineberg, 2004), and has been well studied with the MBT (Table 1). These agents include fluoxetine (Kalariya, Prajapati, Parmar, & Sheth, 2015; Prajapati, Kalaria, Karkare, Parmar, & Sheth, 2011; Uday et al., 2007), fluvoxamine (Harasawa et al., 2006; Matsushima et al., 2009), citalopram (Krass et al., 2010; Li et al., 2006), and paroxetine (Casarotto et al., 2010; Saadat et al., 2006). Furthermore, like the 5-HT releaser fenfluramine (Njung'e & Handley, 1991a),

SSRIs have generally been demonstrated to attenuate burying activity without affecting LMA. Drugs that deplete stores of 5-HT, such as para-chlorophenylalanine (PCPA), also negate the efficacy of fluoxetine (Uday et al., 2007). Further investigations into the function of SSRIs in this context have revealed that blockade of 5-HT_{1A} receptors reduces the efficacy of SSRIs (Casarotto et al., 2010; Harasawa et al., 2006; Ichimaru et al., 1995), whereas, in most cases, blockade of the 5-HT_{2A/C} receptors does not (Ichimaru et al., 1995; however, see Egashira et al., 2013). Nevertheless, given that 5-HT_{2A/C} blockers reversed the attenuating effects of the GnRH analogue leuprolide (Gaikwad et al., 2010) and of 1-

2,5-dimethoxy-4-iodophenyl-2-amino-propane (DOI; a potent 5-HT_{2A/C} agonist; Njung'e & Handley, 1991a) on marble-burying, etiological involvement of the 5-HT_{2A/C} receptors in MBA cannot be excluded. Sigma receptors, believed to be involved in neuroplasticity and cognitive function, have also been proposed to play a role in several affective disorders, although research is currently limited (Ishikawa & Hashimoto, 2010). In this regard, Egashira, Harada, et al. (2007) demonstrated that sigma receptor interactions may also play a role in the therapeutic response caused by SSRIs. Indeed, both the selective sigma-1 receptor antagonist BD 1063 and the non-selective sigma receptor antagonist BD 1047 reversed the attenuating effects of fluvoxamine on MBA, but not that of paroxetine. These findings suggest that unique within-class receptor-dependant mechanisms underlie the actions of fluvoxamine specifically, since activation of the sigma-1 receptor with (+)-SKF 10047 also reduced marble-burying behavior, whereas blockade of the sigma-2 receptor had no effect (Egashira, Harada, et al., 2007).

In line with the above findings, several other drug classes that also affect 5-HT reuptake have also been shown to reduce MBA. Indeed, the predominantly serotonergic tricyclic antidepressant (TCA) clomipramine (Tatsumi, Groshan, Blakely, & Richelson, 1997) reduces burying activity. However, the facts that the dominantly noradrenergic TCAs imipramine and desipramine attenuate MBA (Li et al., 2006; Schneider & Popik, 2007; although see also Ichimaru et al., 1995; Nicolas et al., 2006) and that these agents also target histamine, alpha-adrenergic, and muscarinic receptors (Owens, Morgan, Plott, & Nemeroff, 1997) undermine the predictive validity of these findings within the MBT as a screening paradigm, for both anxiety- and OC-like behavior. Indeed, a possible role for noradrenergic and dopaminergic signaling underlying MBA cannot be excluded, because a number of SNRIs—including duloxetine (Nicolas et al., 2006), milnacipran (Honda et al., 2011; Sugimoto et al., 2007), and venlafaxine (Li et al., 2006)—as well as 5-HT, NA, and DA releasers—including methamphetamine (Saadat et al., 2006), d-amphetamine (Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006), and methylphenidate (Saadat et al., 2006)—have also produced ameliorative effects in the MBT.

The role of specific serotonin receptors In attempting to elucidate the exact mechanisms whereby global increases in 5-HT concentrations modulate MBA, several receptor-specific approaches have been followed. In this regard, the role of the 5-HT₁- and 5-HT₂-receptor classes has been of major interest (Abe et al., 1998; Egashira, Okuno, et al., 2012; Ichimaru et al., 1995; Matsushita et al., 2005; Njung'e & Handley, 1991a).

The nonspecific serotonergic agonist meta-chlorophenylpiperazine (mCPP) has been found to reduce MBA in at least two investigations (Nicolas et al., 2006;

Njung'e & Handley, 1991a), whereas a recent report confirmed marble-burying to respond in a biphasic manner to this agent, with lower doses seemingly inducing burying behavior and higher doses resulting in attenuation (Nardo, Casarotto, Gomes, & Guimarães, 2014). Importantly, the attenuating effect of mCPP does not seem to be reversed by the co-administration of 5-HT-receptor antagonists as has been demonstrated for other 5-HT agonists—for example, DOI (Njung'e & Handley, 1991a). However, mCPP has been found to produce anxiogenic reactions in some individuals (Cornélio & Nunes-de-Souza, 2007; Fox, Hammack, & Falls, 2008) while exacerbating OC episodes in others (Khanna, John, & Reddy, 2001; Zohar, Mueller, Insel, Zohar-Kadouch, & Murphy, 1987). Taking into account the dichotomous effect of mCPP in the MBT, at least some clinical investigations have reported analogous findings with respect to its therapeutic applications in human samples (Pigott et al., 1993). In two other preclinical animal models of OCD—that is, quinpirole-induced compulsive checking and spontaneous stereotypy in the deer mouse—mCPP was also reported to modify compulsive-like behavior routines. Indeed, it reverses compulsive behaviors *induced* by the dopaminergic agonist quinpirole (Tucci et al., 2013), a response that seems unrelated to 5-HT_{2A/C} receptor functioning (Tucci, Dvorkin-Gheva, Johnson, Wong, & Szechtman, 2015). However, in the case of noninduced compulsive-like behaviors, both mCPP and quinpirole have been shown to *attenuate* compulsive-like behaviors (Korff et al., 2008). Although these findings may seem to contradict those reported by Tucci et al. (2013), it is important to note that the latter authors employed quinpirole-induced compulsive behaviors in an attempt to create an *etiologically homologous* population of test subjects, a concept that will be discussed later on.

Substantial evidence for the involvement of the 5-HT_{1A} receptor in marble-burying has been presented. Generally, findings are inconclusive, demonstrating that activation of the 5-HT_{1A} receptor either inhibits burying behavior (Abe et al., 1998; Egashira, Okuno, Matsushita, et al., 2008; Ichimaru et al., 1995; Matsushita et al., 2005; Nicolas et al., 2006; Njung'e & Handley, 1991a; Young et al., 2006), although in certain cases doing so only at doses that also reduced locomotor capability (Abe et al., 1998; Ichimaru et al., 1995; Njung'e & Handley, 1991a) or had no effect at all (Bruins Slot et al., 2008; Li et al., 2006). Possibly of more value for our understanding of the serotonergic mechanisms underlying purportedly aberrant burying activity is the finding that the selective 5-HT_{1A} antagonists WAY 100635 and WAY 100135 block 5-HT_{1A}-induced attenuation of burying behavior (Egashira, Okuno, Matsushita, et al., 2008; Matsushita et al., 2005). Interestingly, as we alluded to earlier, WAY 100635 also seems to reverse the attenuating actions of fluvoxamine (Harasawa et al., 2006) and paroxetine (Casarotto et al., 2010), implicating a specific role for the 5-

HT_{1A} receptor in the actions of the SSRIs in the MBT. That being said, a number of investigations have failed to replicate findings related to the effect of 5-HT_{1A} receptor agonists in the MBT (see, e.g., Li et al., 2006), whereas findings relating to the attenuation of burying behavior have often varied according to dose (Abe et al., 1998; Bruins Slot et al., 2008; Nicolas et al., 2006; Njung'e & Handley, 1991a; Young et al., 2006). These findings therefore cloud the overall conclusions that can be drawn from the collective literature. Moreover, the fact that 5-HT_{1A} agonists, such as 8-OH-DPAT, are used to *induce* compulsive-like behaviors in animals (Alkhatib, Dvorkin-Gheva, & Szechtman, 2013; Yadin et al., 1991), while they also result in anxiogenic effects in the EPM and SIT (Cheeta, Kenny, & File, 2000), supports a conclusion that the marble-burying investigations are not in line with current theories regarding the neurobiology of anxiety and OCD. For example, in contrast to what has been shown in marble-burying investigations, although buspirone appears to be ineffective in treating OCD as a monotherapeutic intervention (Fineberg, 2004; Pigott et al., 1992), it may be of some benefit in combination with an SSRI (Pigott et al., 1992). On the other hand, buspirone has been shown to be very effective in the management of clinical anxiety (Bandelow et al., 2008; Goldberg & Finnerty, 1979; Rickels, 1990).

With respect to the 5-HT₂ receptor subclass, the 5-HT_{2A/C} agonist DOI (Egashira, Okuno, et al., 2012; Njung'e & Handley, 1991a) and the selective 5-HT_{2C} agonist WAY 161503 (Egashira, Okuno, et al., 2012) have been shown to reduce MBA. In addition, co-administration of the 5-HT_{2C} antagonist SB242084 seems also to reverse the attenuating effects of paroxetine, fluvoxamine, and WAY 161503 on MBA (Egashira, Okuno, et al., 2012). However, although the effects of the agonists DOI and WAY 161503 are antagonized by SB242084, ritanserin, and ICI 169,369 (all antagonists of 5-HT₂ receptors; Egashira, Okuno, et al., 2012; Njung'e & Handley, 1991a), the latter two drugs have also been shown to paradoxically *potentiate* the attenuating effect of the SSRIs zimeldine and fluvoxamine on marble-burying behavior (Njung'e & Handley, 1991a). Although the latter effect of ritanserin was not replicated in a later study (Ichimaru et al., 1995), both ritanserin and ICI 169,369 also inhibit marble-burying when administered as monotherapy (Bruins Slot et al., 2008; Njung'e & Handley, 1991a). In addition, Gaikwad et al. (2010) reported no effect of ritanserin administered in the same dose as that used by Njung'e and Handley (1991a; 20 mg/kg). Considering these data, and that ritanserin also reversed the seemingly beneficial effect of leuprolide (a gonadotropin-releasing hormone analogue) on burying behavior (Gaikwad et al., 2010; Uday et al., 2007; Umathe et al., 2008), the relevance of the findings relating to 5-HT₂ receptor function in the burying test is unclear.

Taking the main findings of serotonergic involvement in marble-burying together, the literature is mostly congruent in

showing that 5-HT seems to play a significant role in the neurobiology of marble-burying behavior. Indeed, SSRIs show demonstrable clinical efficacy in the treatment of both anxiety and OCD, as well as generally attenuating marble-burying behavior. However, whereas 5-HT_{2A/C} receptors have been mechanistically implicated in SSRI-induced attenuation of burying behavior in some studies (Egashira, Okuno, et al., 2012), this has not always been the case (Ichimaru et al., 1995). The collective translational value of these findings remains uncertain. Nevertheless, although the MBT as a means to understanding the role of 5-HT in such behavior has yielded inconsistent results, the test may be useful in studies of elicited behavior in which the involvement of specific serotonergic mechanisms may be investigated (Kedia & Chattarji, 2014). Furthermore, the translational relevance of the test to explain anxiolytic and anticomulsive responses following acute increases in serotonergic signaling remains poorly defined.

Dopaminergic system involvement in marble-burying

Considering the role of DA antagonists in treatment augmentation strategies for OCD (Fineberg, 2004), several compounds in this class, including amisulpiride (Bruins Slot et al., 2008), haloperidol (Broekkamp et al., 1986; Bruins Slot et al., 2008; Matsushita et al., 2005; Nicolas et al., 2006), L-741626 (Egashira, Okuno, Matsushita, et al., 2008), olanzapine (Bruins Slot et al., 2008; Egashira, Okuno, Matsushita, et al., 2008), perospirone (Matsushita et al., 2005), perphenazine (Nicolas et al., 2006), quetiapine (Egashira, Okuno, Matsushita, et al., 2008), remoxipride (Bruins Slot et al., 2008), risperidone (Bruins Slot et al., 2008; Li et al., 2006; Matsushita et al., 2005; Torres-Lista et al., 2015), aripiprazole (Gaikwad & Parle, 2011), and thioridazine (Broekkamp et al., 1986) have been investigated in the MBT, generally resulting in attenuation of burying behavior, with few exceptions (Honda et al., 2011). All of the said compounds have been administered as monotherapy in animals demonstrating inherent, nonprovoked burying behavior. These findings must therefore be viewed in light of DA's relevance within the context of OCD and anxiety. Although DA antagonists may be of value in treatment augmentation strategies with SSRIs in cases of treatment-refractory symptoms (Abramowitz et al., 2009; Dougherty et al., 2004; Fineberg, 2004), antidopaminergic treatment on its own is of negligible value in the treatment of anxiety disorders and OCD; DA antagonists are, however, often added to anxiolytic regimens to treat co-morbid symptoms of psychosis or in treatment resistant instances (Bandelow et al., 2008). Moreover, DA potentiators—that is, receptor agonists, including quinpirole (Egashira, Okuno, Abe, et al., 2008), pramipexole (Jimenez-Gomez et al., 2011), and PD168077 (Bruins Slot et al., 2008); DA reuptake inhibitors, including GBR 12909 (Saadat et al., 2006), bupropion (Honda et al.,

2011), and amantadine (Egashira, Okuno, Harada, et al., 2008); as well as DA precursors, including carbidopa (Njung'e & Handley, 1991b)—administered as monotherapy have also been demonstrated to attenuate burying activity. These findings are especially interesting, given that quinpirole have been used to *induce* OC-like behaviors in animal models of OCD (Albelda & Joel, 2012a; Szechtman et al., 1998). However, the effect of dopaminergic manipulation and its modulation of neuropsychiatric symptomology may be highly related to the context of investigation. In fact, while quinpirole induces compulsive-like checking behavior in rats (Szechtman et al., 1998), it also reduces naturally occurring OC-like stereotypy in the deer mouse (Korff et al., 2008).

Taken together, both dopaminergic and antidopaminergic compounds attenuate burying behavior, albeit variably so (Tables 1, 2, and 3), with few exceptions (Honda et al., 2011). These findings have invariably been reported following the application of tests of unprovoked marble-burying. Therefore, any conclusions drawn with respect to the neurobiological involvement of DA in “so-called” anxiety- or OC-like behaviors are speculative at best.

Glutamatergic involvement in marble-burying behavior

A number of findings support a role for altered glutamatergic signaling in both anxiety (Bergink, van Megen, & Westenberg, 2004; Cortese & Phan, 2005) and OCD (Abramowitz et al., 2009), constituting proof of principle for investigating the effects of drugs targeting glutamate receptors in the MBT. Apart from the attenuating effects of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor potentiators on burying behavior (Iijima et al., 2010; however, see also Egashira, Okuno, Harada, et al., 2008), the findings largely demonstrate that *N*-methyl-D-aspartate (NMDA) and metabotropic glutamate receptor (mGluR) antagonism attenuate burying behavior (Dixit et al., 2014; Egashira, Okuno, Harada, et al., 2008; Iijima et al., 2010; Nicolas et al., 2006; Shimazaki et al., 2004). Furthermore, fenobam, an mGluR 5 allosteric modulator, has also been shown to reduce burying activity (Nicolas et al., 2006). Interestingly, the response of antiglutamatergic compounds seem specific to the modulation of baseline glutamate signaling, since monotherapy with glutamate agonists does not seem to exacerbate burying behavior (Shimazaki et al., 2004). Furthermore, since animal studies have indicated NMDA receptor involvement in both the acquisition and extinction of fear responses, the modulation of MBA and inhibition of related fear responses by NMDA receptor antagonists seemingly supports a role for neophobic triggers in marble-burying behavior (Cortese & Phan, 2005). Indeed, mGluR modulation has also been found to elicit anxiolytic action in several other animal behavior paradigms (Cortese & Phan, 2005). Given

the evidence that agmatine blocks NMDA receptors (Yang & Reis, 1999), the administration of agmatine may also interact with this neurotransmission system in this manner (Dixit et al., 2014; Gawali et al., 2016; Krass et al., 2010; although see also “Miscellaneous pathways involved in marble-burying behavior” section).

Marble-burying and GABAergic signaling

The benzodiazepine class of drugs forms a core component in the short-term management of multiple anxiety disorders (Bandelow et al., 2012; Bandelow et al., 2008). Although they have no demonstrable effect in the treatment of OCD (Abramowitz et al., 2009; Fineberg, 2004), the benzodiazepines, which modulate the effects of the inhibitory neurotransmitter GABA via binding to GABA_A receptor complexes (Pym, Cook, Rosahl, McKernan, & Atack, 2005), have been studied extensively in the MBT. However, this work involved MBA as a screening test for anxiolytic drug action rather than applying the test itself as a framework in which to study the mechanisms underlying anxiety. Indeed, clonazepam (Broekkamp et al., 1986), alprazolam (Nicolas et al., 2006), and diazepam (Abe et al., 1998; Broekkamp et al., 1986; Kinsey et al., 2011) have all been studied in the MBT, mostly yielding results that supported the test's validity as a screening tool for anxiolytic drug action. However, many of these findings were often paired with significant locomotor suppression across most doses tested (Abe et al., 1998; Nicolas et al., 2006; Schneider & Popik, 2007; Young et al., 2006). Hence, follow-up investigations attempted to distinguish between actual anxiolytic responses and the coincidental effects of locomotor suppression by means of selectively targeting GABA receptors. These studies reported inconsistent results: The GABA_A agonists muscimol (which binds to the GABA-binding site of the GABA_A receptor complex) and meprobamate (binding to the barbiturate-binding site of the GABA_A receptor complex), as well as the GABA_B agonist baclofen, have all been demonstrated to reduce marble-burying (Broekkamp et al., 1986; Egashira et al., 2013). However, the same is true for the *anxiogenic* β -carboline, methyl beta-carboline-3-carboxylate (β -CCM), an inverse GABA_A benzodiazepine receptor agonist (Jimenez-Gomez et al., 2011). Furthermore, although the effects of β -CCM have also been shown to be associated with significant locomotor suppression (Jimenez-Gomez et al., 2011), other studies have failed to demonstrate changes in either MBA or LMA using the β -carboline derivative, ethyl-beta-carboline-3-carboxylate (β -CCE; Njung'e & Handley, 1991b). That said, the attenuating effect of GABA_A receptor modulation on burying behavior may be subject to activation-specific GABA_A receptor complexes, since the partial GABA_A agonist bretazenil, which binds to GABA_A complexes irrespective of the alpha subunit expressed (Pym et al., 2005), does not seem to affect the burying response

(Li et al., 2006); however, further investigation is warranted. Moreover, whereas the administration of the competitive GABA_A antagonist bicuculline alone fails to influence burying behavior, it prevents valproate- and muscimol-induced reductions in MBA (Egashira et al., 2013). In congruence with this finding, flumazenil, another GABA_A benzodiazepine receptor antagonist, also prevented the attenuating effects of benzodiazepines on MBA (De Almeida et al., 2014). Not surprising, though, is that given baclofen's use as a skeletal muscle relaxant, the GABA_B-mediated reductions of MBA induced by this compound are associated with notable impairments in locomotor capability (Egashira et al., 2013). However, quite remarkable is that the barbiturate phenobarbital failed to reduce marble-burying behavior (Li et al., 2006).

That compounds targeting GABAergic neurotransmission are effective against anxiety but not OCD somewhat clouds the interpretation of data relating to anxiolytic drug action generated in the MBT. It is common practice to manipulate a certain behavioral test pharmacologically, to allow it to constitute a valid framework that resembles a specific neurocognitive construct. This allows the behavioral test to be deployed as a screening tool for drug action following receptor-targeted interference. With respect to anxiety-related investigations, it would therefore be valuable to administer known anxiogenic substances and to quantify baseline MBA; subsequently, purported anxiolytic compounds may be administered in order to establish whether such pharmacological manipulations are successful, directly relating the behavioral outcome to a specific mechanism. However, given that the majority of investigations have employed unprovoked burying behavior, it is not possible to make any concrete deductions with respect to either anxiety or OCD.

Noradrenergic responses in the MBT

Although the noradrenergic system is typically not implicated in the neurobiology of OCD, it may have some role to play in the manifestation of anxiety (Hoehn-Saric & McLeod, 1988; Tanaka, Yoshida, Emoto, & Ishii, 2000). In this regard, a handful of studies have investigated the possible role of noradrenergic manipulation in the MBT. Indeed, the noradrenergic tricyclic antidepressant imipramine (Broekkamp et al., 1986; Li et al., 2006), the SNRI nisoxetine (Li et al., 2006), and the nonselective NA, 5-HT, and DA releasing agents (Green et al., 2003; Rowley, Pinder, Kulkarni, Cheetham, & Heal, 2015) methylphenidate (Saadat et al., 2006), d-amphetamine (Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006), and methamphetamine (Saadat et al., 2006) all reduce the expression of MBA.

NA has been demonstrated to influence anxiety-like behaviors in preclinical studies; levels are typically increased in a number of brain regions, including the hippocampus and

amygdala, in response to stressful events (Brunello et al., 2003; Tanaka et al., 2000). The neurobehavioral effects of such increases can typically be prevented by the administration of compounds not necessarily related to noradrenergic functioning—for instance, the benzodiazepines and opioids (Tanaka et al., 2000). Furthermore, anxious behaviors during these tests can be further exacerbated by drugs inducing NA release (Tanaka et al., 2000). Therefore, and also considering that potentiating noradrenergic signaling will also generally exacerbate OCD symptoms (Coşkun, 2011; Serby, 2003; Shakeri et al., 2016), it is somewhat confounding that noradrenergic-bolstering agents are effective at *reducing* MBA (Jimenez-Gomez et al., 2011; Li et al., 2006; Saadat et al., 2006). This is especially problematic given that they have been administered acutely prior to execution of the MBT. Indeed, these effects may possibly be attributed to concomitant increases in the activity of other monoamines—that is, DA, in the case of amphetamine-like drugs (Green et al., 2003).

Mood stabilizers and anticonvulsants

Although anticonvulsants are not typically indicated for the treatment of either OCD (Abramowitz et al., 2009; Fineberg, 2004) or anxiety (Bandelow et al., 2008), carbamazepine, lamotrigine, and valproate have indeed been shown to attenuate MBA. Interestingly, lamotrigine displayed strain-dependant treatment effects, in that it was effective in ICR but not ddY mice, supporting the findings previously discussed pertaining to strain-to-strain variation in nonmanipulated MBA (Egashira et al., 2013; Nicolas et al., 2006; Thomas et al., 2009). In fact, should such findings be reproducible, they will add weight to the argument for the standardization of strain selection in the MBT (see also “Final summary and recommendations for using the MBT” section). The aforementioned anticonvulsant drugs modulate a number of physiological processes, including the facilitation of GABAergic neurotransmission, blockade of sodium channels, and glutamate receptors, in addition to stimulating dopaminergic and serotonergic neurotransmission (Ambrósio, Soares-da-Silva, Carvalho, & Carvalho, 2002; Egashira et al., 2013; Perucca, 2002), complicating deductions regarding their specific therapeutic mechanisms. However, a role for GABAergic neurotransmission in their mechanism has been demonstrated by the ability of the GABA_A antagonist bicuculline to antagonize the attenuating effects of valproate in the MBT (Egashira et al., 2013). Oddly enough, the mood stabilizer lithium does not have a demonstrable effect on MBA, despite lowering LMA significantly (Egashira et al., 2013). This lack of effect may be due to lithium's known slow onset of action, which ranges from between 6 and 10 days, for the reduction of manic symptoms in clinical bipolar disorder, to more than 6 weeks, for the attenuation of depressive

symptoms (Malhi, Adams, & Berk, 2009); therefore, a single acute administration 30 min prior to testing (Egashira et al., 2013) is arguably insufficient to elicit observable change (Jope, 1999). Changes to LMA may be explained by transient changes to excitatory and inhibitory neurotransmission processes (Jope, 1999).

Cannabinoid receptor involvement in marble-burying

The endocannabinoid system represents a relatively novel array of therapeutic targets for drug discovery. The cannabinoid receptors (CB₁ and CB₂), the principal effectors of the system (Howlett et al., 2002), are typically activated by the endogenous cannabinoid anandamide, whereas a number of agonists and antagonists for these receptors have been characterized to date (Ameri, 1999; Pertwee, 1997). Although both receptors are widely distributed, the CB₁ receptor has been extensively studied for its CNS effects, whereas the CB₂ receptor is largely expressed on immune cells in the periphery (Ameri, 1999; Howlett et al., 2002). Presynaptically located CB₁ heteroreceptors function to reduce the release of a range of neurotransmitters, including glutamate and GABA (Schlicker & Kathmann, 2001), whereas acetylcholine and dynorphin release are also affected. Furthermore, neurotransmitter release may be manipulated by CB₁ agonists at multiple levels of control, such as the *bolstered* release of DA in certain brain regions, by means of removal of the inhibitory effects of tonically active GABAergic neurons following activation of presynaptic CB₁ receptors (Howlett et al., 2002; Schlicker & Kathmann, 2001).

A number of significant findings considering the role of cannabinoid-like effects in the MBT have been made, with agonists of cannabinoid receptors exerting dose-dependent actions on MBA. Anandamide (Umathe et al., 2012) and WIN55,212-2 (Gomes et al., 2011), both of which are cannabinoid receptor 1 (CB₁) agonists, attenuate MBA at lower doses, while exacerbating such behavior at higher doses. This seems to also be true for AM404 and URB597, which act to facilitate anandamide neurotransmission by inhibiting its reuptake or preventing its metabolism (Umathe et al., 2012), respectively. It is hypothesized that such a bimodal effect of anandamide can be attributed to the activation of transient receptor potential vanilloid type 1 (TRPV1) channels, which occurs when anandamide concentrations increase above those required for cannabinoid receptor stimulation (Umathe et al., 2012). Furthermore, these findings have been corroborated by the demonstration that capsazepine, a TRPV1 antagonist, reduces marble-burying, whereas capsaicin, a TRPV1 agonist, elicits pro-compulsive effects in the test (Umathe et al., 2012). Also, antagonism of the TRPV1 receptor by capsazepine blocks the pro-compulsive effects of high dose anandamide, AM404 and URB597, but has no effect

when these compounds are administered in lower doses, indicating a modulatory role for TRPV1 receptor activation underlying pro-compulsive responses. Considering receptor-specific effects, THC (Δ^9 -tetrahydrocannabinol), a nonselective cannabinoid receptor agonist, and cannabidiol (CBD), a nonselective indirect *antagonist*, also appear to also reduce MBA (Kinsey et al., 2011). Importantly, irrespective of receptor selectivity, the attenuating effects of CBD (Casarotto et al., 2010), anandamide (Umathe et al., 2012), AM404 (Gomes et al., 2011; Umathe et al., 2012), WIN55,212-2 (Gomes et al., 2011), and URB597 (Gomes et al., 2011; Umathe et al., 2012) are blocked by the CB₁ receptor antagonist AM251 (Casarotto et al., 2010; Gomes et al., 2011; Umathe et al., 2012). Since CBD itself also functions as an antagonist of the cannabinoid CB₁ and CB₂ receptors (Pertwee, 2008), the fact that it attenuates MBA on its own, and that such attenuation can be blocked by another antagonist, in the form of AM251, seems confounding. However, the attenuating effect of CBD on marble-burying behavior can possibly be ascribed to the decreased hydrolysis and reuptake of anandamide, and subsequent stimulation of CB₁ receptors, following the administration of CBD (Bisogno et al., 2001; Casarotto et al., 2010). Importantly, crosstalk between cannabinoid and serotonergic neurotransmission in the marble-burying response has been suggested by findings indicating that the co-administration of subeffective doses of CBD and fluoxetine reduces burying activity (Nardo et al., 2014). Furthermore, CBD was also able to reverse mCPP-induced MBA (Nardo et al., 2014). Finally, endogenous ligands of the endocannabinoid receptors also appear to modulate MBA, since inhibition of their catabolism also reduces MBA; this effect can also be blocked by CB₁ antagonist rimonabant (Kinsey et al., 2011).

The above findings are presented within the context of OC-like behavior; however, they must be viewed in light of the fact that endocannabinoid-like compounds do indeed exhibit anxiolytic action, particularly via CB₁ receptor activation (Haller, Varga, Ledent, & Freund, 2004; Rey, Purrio, Viveros, & Lutz, 2012). Anxiogenic effects can be precipitated by CB₁ blockade (Haller et al., 2004; Navarro et al., 1997), but similar manipulations can *also* reduce anxiety-like manifestations (Casarotto et al., 2010; Haller, Bakos, Szirmay, Ledent, & Freund, 2002). Additionally, this CB₁-mediated biphasic anxiety response is well known, and has in fact been replicated in other animal anxiety tests (Haller et al., 2004; Moreira & Wotjak, 2009; Rey et al., 2012). In this respect, the MBT appears to accurately mimic the behavioral manifestations of direct and indirect endocannabinoid receptor manipulation reported in other tests, so this test may be of particular interest for further study of drugs modulating this system. That said, because only some proof of concept exists that warrants further investigation of the effects of cannabinoid receptor modulation in OCD (Casarotto et al., 2010), the translational

relevance of these results with respect to OCD remains to be established.

Miscellaneous pathways involved in marble-burying behavior

A number of miscellaneous systems have been investigated in the MBT. Since the core focus of the present article is on anxiety and OCD, and considering that to date none of these have shown any clear and demonstrable role in current approaches to the treatment of either condition, they will only be briefly summarized.

Nitric oxide (NO) involvement in the MBA has been implicated in a few studies. It has been shown that administration of the NO precursor L-arginine, increases MBA (Gawali et al., 2016), while it also reverses the attenuating effect of SSRI administration on burying behavior (Krass et al., 2010). Furthermore, many inhibitors of NO synthesis—such as 7-nitroindazole (7-NI; Gawali et al., 2016; Krass et al., 2010), N^G-nitro-L-arginine methyl ester (L-NAME; Gawali et al., 2016), and 1-(2-trifluoromethylphenyl) imidazole (TRIM; Krass et al., 2010)—have demonstrated potential for reducing burying activity, implying a possible role for NO in the manifestation of MBA. In line with these findings, agmatine, a modulator of glutamatergic NMDA receptors that also decreases central NO activity, reduces MBA in a manner that can be reversed by L-arginine (Gawali et al., 2016). These findings are especially interesting with respect to OCD, in that previous reports had indicated that elevated NO levels in the rat brain suppress 5-HT activity in those same regions, whereas NO inhibitors overturn this effect (Gawali et al., 2016; Segieth, Pearce, Fowler, & Whitton, 2001; Wegener, Volke, & Rosenberg, 2000). Moreover, that agmatine has also demonstrated anxiolytic action in the EPM and SIT supports the abovementioned findings, in so far as the MBT is purported to represent both an anxiety-like and OC-like constructs (Gong et al., 2006). In physiological terms, elevated NO concentrations increase the activity of guanylyl cyclase, which increases cyclic guanosine 3'5'-monophosphate (cGMP) levels, an important second messenger known to interact with a number of neurotransmission processes (Feil & Kleppisch, 2008), most notably, those that also have established roles in OCD—for instance, GABA, glutamate, DA, and 5-HT. Indeed, NO may modulate 5-HT, GABA, and glutamate release (Kaehler et al., 1999; Sandor, Brassai, Pliskas, & Lendvai, 1995; Spiacci, Kanamaru, Guimaraes, & Oliveira, 2008), and NO modulation has been demonstrated to elicit anxiolytic and antidepressant effects (Spiacci et al., 2008). As such, proof of concept exists for further investigations of NO manipulation in preclinical and clinical studies of anxiety and OCD.

Melanin-concentrating hormone (MCH) appears to modulate MBA, since administration of the hormone itself reduces this behavior. Oxytocin administration also reduces the

behavior, since many MCH neurons express oxytocin receptors that influence their activity levels. Finally, blockade of the MCH receptor with antagonist GW803430 prevents the attenuating actions of both the aforementioned hormones (Sanathara et al., 2018). Oxytocin has been linked to anxious behavior (Neumann & Landgraf, 2012).

Although antagonism of neurokinin receptor 1 (NK₁) has shown promise as a potential anxiolytic in both preclinical (Santarelli et al., 2001) and clinical (Tillisch et al., 2012) studies, the inhibition of MBA by NK₁ antagonists—for example, GR205,171 and RP67,580—is associated with significant locomotor impairment (Millan et al., 2002). It is therefore likely that the MBT is not sensitive enough to reveal possible anxiolytic responses following antineurokinin compounds.

Findings related to the effects of first-generation antihistamines—for instance, chlorpromazine (Abe et al., 1998; Broekkamp et al., 1986; Li et al., 2006; Nicolas et al., 2006), cyproheptadine (Njung'e & Handley, 1991a), and diphenhydramine (Broekkamp et al., 1986)—in the MBT are mixed. Although they have demonstrated positive effects in some investigations (Abe et al., 1998; Broekkamp et al., 1986; Li et al., 2006; Nicolas et al., 2006; Njung'e & Handley, 1991a), they have failed to do so in others (Schneider & Popik, 2007). Furthermore, since most of these compounds function as multipotent competitive antagonists at a number of receptors, including the noradrenergic alpha, dopaminergic D₁ and D₂, and cholinergic M-receptors (Owens et al., 1997), the translational relevance of these findings is difficult to interpret within the context of anxiety and OCD. Furthermore, since first-generation antihistamines cause significant motor impairment and sedation, their effects in the MBT are often confounded by parallel reductions in locomotor competence (Abe et al., 1998; Njung'e & Handley, 1991a; Nicolas et al., 2006).

What may be more important within the context of the present article is that, in addition to the major receptor systems discussed, a variety of compounds have been put forward as valid alternatives for reducing MBA, and thus for further study within the context of anxiety and OCD. These include *N*-acetyl-L-cysteine (NAC; Egashira, Shirakawa, et al., 2012) and ascorbic acid (De Almeida et al., 2014), which connects with evidence for redox disturbances in human OCD and animal models (Guldenpfennig, Wolmarans, du Preez, Stein, & Harvey, 2011; Szechtman et al., 2017); calcium channel blockers (Egashira, Okuno, Abe, et al., 2008), which connects with evidence of the efficacy of mood stabilizers in OCD (Egashira et al., 2013); anticholinergic drugs, such as atropine (Broekkamp et al., 1986; Nicolas et al., 2006) and scopolamine (Broekkamp et al., 1986); cholinergic compounds, such as organophosphates (Savy et al., 2015); novel plant extracts (Dey et al., 2016; Kalariya et al., 2015; Skalisz, Beijamini, & Andreatini, 2004); and morphine (Nicolas et al., 2006). However, the relevance, usefulness, and translational contribution of these findings must be questioned, in light of the

concerns raised with respect to the methodologies followed in the execution of the MBT and the uncertain neuropsychological construct resembled by the MBT that we noted earlier. Suffice to say, with the exception of NAC, which has demonstrated a possible augmentation role in SSRI refractory OCD (Lafleur et al., 2006) and as a possible treatment for OC-spectrum disorders such as trichotillomania (de L. T. Vieira, Lossie, Lay, Radcliffe, & Garner, 2017; Özcan & Seckin, 2016), few of these findings have resulted in any clinical application. Although they may possibly contribute to our understanding of the mechanisms underlying MBA, this remains unlikely, given that these agents have shown little demonstrable effect with any other behavioral screening tools for either anxiety- or compulsive-like behaviors.

The role of pharmacologically induced burying behavior in the MBT

As we alluded to earlier, animal behavioral tests that are not only responsive to ameliorative therapeutic interventions, but additionally show detectable behavioral changes in response to exacerbating pharmacological manipulation (File & Seth, 2003; Tanaka et al., 2000), are useful for elucidating the specific neurobiological mechanisms underlying proposed anxiety- and compulsive-like behaviors. Considering that the MBT is indeed sensitive to anxiogenic (Kedia & Chattarji, 2014; Umathe et al., 2009) and pro-compulsive (Tucci et al., 2013) intervention and that it is applied as a measure of anxiety- and compulsive-like behavior, several investigations have attempted to bolster burying responses by administering drugs with known anxiogenic or pro-compulsive properties. However, the results have been inconsistent. For example, in terms of classic anxiogenic compounds, several have been shown to *reduce* marble-burying behavior. These include yohimbine (Nicolas et al., 2006; Njung'e & Handley, 1991b), β -carbolines (Jimenez-Gomez et al., 2011), and amphetamine (which are often paired with significant increases in LMA; Jimenez-Gomez et al., 2011; Li et al., 2006; Nicolas et al., 2006; Saadat et al., 2006). Furthermore, even this response is inconsistent, with some anxiogenic agents, such as FG-7142 (Nicolas et al., 2006) and β -carbolines (Njung'e & Handley, 1991b), not modulating the burying response at all. Furthermore, as we alluded to earlier, drugs known to induce compulsive-like behavioral persistence in animal models (which incidentally is what also characterizes MBA), such as quinpirole (Egashira, Okuno, Harada, et al., 2008; Szechtman et al., 1998; Tucci et al., 2013) and 8-OH-DPAT (Bruins Slot et al., 2008; Ichimaru et al., 1995; Yadin et al., 1991), also elicited ameliorating responses. Also, the DA agonist pramipexole has demonstrated pro-compulsive effects in human studies, yet it reduces MBA in rodents (Jimenez-

Gomez et al., 2011). Although demonstrating *sensitivity* to such interventions per se may potentially contribute to our understanding of the underlying neurobiology of MBA—that is, confirming a role for altered dopaminergic and serotonergic signaling—the indiscriminate and variable between-laboratory responses to the same compounds or drug classes negate the translational value of such findings. Indeed, reliability and reproducibility of experimental paradigms according to similar methodologies will be crucial before it will be possible to draw collective translational conclusions (Varga, Hansen, Sandøe, & Olsson, 2010). As such, given the data summarized above, marble-burying cannot be regarded as a reliable screening test for any *specific* neuropsychological construct.

Timing and duration of treatment

An important consideration in anxiety and OCD studies, and one that applies to all forms of treatment, concerns the timing and duration of a pharmacotherapeutic intervention. Apart from demonstrating the involvement of several neurotransmitter systems in MBA, few studies have addressed the fact that, with the exception of such drug classes as the benzodiazepines in the treatment of anxiety-like behavior, acute interference usually exacerbates rather than attenuates anxiety-like behavior (Bandelow et al., 2008; Fineberg, 2004). Indeed, with only a few exceptions (De Almeida et al., 2014; Ichimaru et al., 1995; Njung'e & Handley, 1991a; Savy et al., 2015; Taylor et al., 2017; Torres-Lista et al., 2015; Umathe et al., 2012; Wolmarans et al., 2016), most investigations into MBA have employed acute treatment 30 min prior to the test (Tables 1, 2, and 3). For instance, although a role for 5-HT has been implicated in the expression of MBA, the translational relevance of anxiolytic and anticomulsive responses subsequent to acute manipulations of neurotransmission has not been explained. Indeed, clinical responses to SSRIs typically require up to eight weeks for both OCD (Goddard et al., 2008) and anxiety (Bandelow et al., 2008). As such, studies testing MBA in response to chronic treatment with such agents will be vital for it to be considered a reliable measure of anxiolytic and/or anticomulsive drug action. Nevertheless, the effects of SSRIs on MBA have in some cases been shown to taper off during repeated administration (Njung'e & Handley, 1991a), while being maintained (Ichimaru et al., 1995; Umathe et al., 2012), or even demonstrating diminution of effect over 28 days of treatment (Wolmarans et al., 2016), in others. Reports concerning chronic diazepam treatment have also reported both successful (Taylor et al., 2017) and unsuccessful (Ichimaru et al., 1995) outcomes following sustained therapy. Similarly, most agents acting on the endocannabinoid system lose their efficacy after two weeks, whereas drugs such as the TRPV1 antagonists maintain suppression of MBA over this

period (Umathe et al., 2012). These unexplained findings warrant further study.

Concluding remarks on treatment responses in the MBT

Given the body of evidence presented here, it is clear that the MBT, as reported up to this point, has “responded” to a broad range of pharmacological interventions, some of which are in line with other preclinical and clinical evidence. That said, various compounds have also demonstrated attenuating effects, for which no current explanation of its contextual mechanism of action have been proposed. Furthermore, the evidence is replete with contradictions, in terms of the effects of specific agents, effective dosages, paradoxical effects of anxiogenic drugs, and divergent findings regarding agonist/antagonist pairings, as we highlighted in earlier sections. Since the MBT is often regarded as a relatively straightforward procedure to apply as a test for anxiolytic and anticomulsive drug action, we propose that such divergent findings have been influenced by the methodological inconsistencies summarized in “Digging, burrowing, and burying as natural behaviors” section, as well as by the general lack of standardization regarding the MBT as a whole. However, on the one hand, *aberrant* naturally occurring marble-burying behavior appears to hold some value as a potential *model* for neophobia or compulsive behavior. On the other hand, we posit that anxiety- or compulsive-like behaviors that are induced by pharmacological manipulation and are accurately highlighted by changes in normal burying behavior, as measured according to the criteria suggested for each condition in “Final summary and recommendations for using the MBT” section, could be of value as high-throughput *screening tests*, whereby any drugs that test positive may then be pursued further and characterized in more robust, pathology-driven models. Also, considering the potential ease, low cost, and high throughput capacity of the test, the MBT may potentially be ideal for examining the behavioral effects of specific receptor manipulations. This approach could allow for the *identification* of novel drug–receptor interactions that might be of relevance for anxiety and OCD, as has been demonstrated with respect to sigma receptor involvement (Egashira, Harada, et al., 2007), the various endocannabinoid-targeting drugs (Casarotto et al., 2010; Gomes et al., 2011; Kinsey et al., 2011; Umathe et al., 2012), antioxidants (Egashira, Shirakawa, et al., 2012), and drugs regulating NO synthesis (Gawali et al., 2016; Krass et al., 2010).

Final summary and recommendations for using the MBT

The body of literature reviewed in the present article has highlighted a number of between-laboratory inconsistencies.

To further the collective translational value of findings reported from investigations into marble-burying activity, and because methodological differences may have fundamentally contributed to the discrepancies in the findings reported, we suggest the alignment of methods, but at the same time consider that context-specific experimental design will be crucial. Briefly, we make the following recommendations with respect to execution of the MBT:

- Researchers should adopt a fixed size (e.g., 15 mm for mice, 20 mm for rats), number, and manner of placement of marbles in specific circumstances that are standardized for different species, cage sizes, and testing paradigms—that is, one-zone versus two-zone configurations and anxiety- versus compulsivity-related investigations.
- Burying substrates of a denser nature (i.e., river sand) should be used, since this prevents incidental settling of marbles during typical behavioral routines. Furthermore, irrespective of the neuropsychological construct investigated, the test subjects should be exposed to the burying substrate in home cages for at least 24 h prior to testing, to avoid novelty-induced burying activity—this will ensure that marbles are the only novel stimuli introduced in the testing paradigm. Furthermore, regardless of the substrate used, the average density and particle diameter thereof must be reported, to ensure adequate between-laboratory repeatability.
- Video tracking of the test procedure should form a core component of marble-burying investigations. Visual scrutiny of video should accurately confirm marble-directed behavior, as opposed to normal cage exploratory activity that results in coincidental covering of marbles. Furthermore, time spent on either side of a two-zone test paradigm should be applied as an indicator of avoidance or engagement behavior.
- Locomotor activity should always be accounted for. We propose that a separate group of animals be used for this objective, since simultaneous scoring of locomotor ability, typically quantified as the total distance traveled, may be confounded by marble-directed preoccupation during the test.
- The arena size should be standardized per species, whereas the spacing of marbles in arenas should allow sufficient room for avoidance, and therefore be sensitive to measuring behaviors related to *voluntary interaction*. The standard housing cages often used for mice and rats represent an ideal point of departure. Dimensions of 35 × 20 × 13 cm and 40 × 35 × 21 cm and for mice and rats, respectively, may be suitable. Naturally, interlaboratory differences in equipment will be apparent, but efforts should be made to minimize such differences.

- Since scoring of the test is arguably subjective, we recommend that two blind scorers be involved in the testing procedures. Applying score averages or any other viable method of normalizing scores will contribute to the validity and value of the assigned score, especially when combined with analogous use of burying substrates and analyses of video recordings. To ensure accurate between-laboratory methodological repetition, investigators should also describe in detail how their scoring procedures are carried out.
- Since different levels of inherent digging and MBA for different species and strains have been demonstrated, we recommend selecting specific strains for investigations of specific neurocognitive constructs, except in scenarios in which existing animal models of OCD or anxiety are scrutinized in the MBT for the presence of *additional* behavioral phenotypes—for example, deer mice (Wolmarans et al., 2016). The selected strains should demonstrate consistent burying behavior that is subject to modification by either known exacerbating or attenuating interventions. In this regard, it seems that C57BL mice may represent an ideal strain for such testing, since it has demonstrated sensitivity to mild anxiogenic behavioral interventions such as restraint, together with an apparent propensity to both avoid marbles and bury them when close by, indicating a likely neophobic behavioral phenotype (Kedia & Chattarji, 2014; Nicolas et al., 2006).
- Since the majority of reported anxiety studies have employed natural, noninduced burying behavior, the only possible anxiety-related trigger for burying activity has been the introduction of marbles as a novel stimulus. In this regard, a two-zone paradigm, in which the animal has the ability to avoid exposure to the marbles completely or can intentionally engage in active burying behaviors, is advised. Furthermore, it would be valuable to demonstrate that neophobic responses abate over time; hence, repeated testing over consecutive days is also advised.
- Since OC symptoms are inflexible, persistent, and directed at specific actions, these traits should ideally be emulated in the MBT, either with respect to natural behaviors or where such activity is artificially induced. In this regard, the use of a two-zone paradigm is crucial, in that (1) this allows the animal the choice of engagement, thereby emulating goal-directed responses, and (2) the test will be discriminative with regard to anxiety-related constructs—for example, avoidance. Furthermore, assessing burying behaviors over repeated trials both before (for baseline scoring) and after treatment will also indicate whether the burying construct is subject to habituation over time or whether it persists, thereby more accurately mimicking compulsive-like repetition.

Conclusion

The MBT is often applied as a model of anxiety- and compulsive-like behavior, as well as as a preclinical screening test to recognize potential anxiolytic or anticomulsive drug action. However, the findings from marble-burying investigations as they have been reported up to now have been inconsistent and often contradictory, and therefore have not contributed much in terms of translational usefulness. Although the present review has sought to highlight the various methodologies followed, we have also explained why the test in itself has not been used to its full potential. Furthermore, on the basis of anxiety- and OC-related theory, we have argued that certain methodological specifics are important for aligning the MBT as closely as possible to the illness under study. In this regard, we propose that the testing of anxiety or OCD should be assessed *after* anxiogenic or pro-compulsive interventions to facilitate marble-burying, be they behavioral (Kedia & Chattarji, 2014) or pharmacological (Nardo et al., 2014; Sanathara et al., 2018; Tucci et al., 2013; Umathe et al., 2012), so as to examine the effect of treatment in a more robust simulation of anxiety and compulsivity. With regard to using the MBT as a *model* of neophobia and OCD, emphasis should *ideally* rest on the demonstration of three key observations: namely, (1) that interactions with marbles are goal-directed and voluntary; (2) that MBA abates over time, with respect to anxiety, or persists over repeated trials in control groups, with respect to OCD; and (3) that the effects of anxiolytic and anticomulsive drugs are present only after the course of a chronic administration schedule, except in the case of drugs that could be of value in the acute treatment of anxiety. Furthermore, we have elaborated on the numerous pharmacological interventions that have been tested, described how these studies have extended (or not extended) understanding, and highlighted possible reasons why the results obtained have been counterproductive. That said, we reiterate the *potential* utility of appraising burying behavior, as either a possible model or a test for the identification of anxiety- or compulsive-like constructs and novel drug–receptor interactions, and as an instrument to screen for drugs that target novel physiological sites that have already demonstrated, or have been designed for, possible anxiolytic or anticomulsive activity, provided that a sound and contextually relevant experimental design is followed. Suggestions and recommendations for such methodologies have also been provided.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interest.

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