Too depressed to swim or too afraid to stop? A reinterpretation of the forced swim test as a measure of anxiety-like behavior.

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Depression is one of the most common forms of mental illness. Despite decades of research, the mechanisms underlying this disorder remain unknown. To help elucidate the pathophysiology of depression, researchers are developing animal models of psychiatric disorders, including depression, and assessing for mood-related phenotypes. There are a limited number of behavioral assays available to assess depression-like behaviors in rodents but by far the most common is the forced swim test (FST). Although the FST is considered the gold standard for studying depression-like behaviors there are strong reasons to question the interpretation that immobility represents 'despair' and escape-directed behaviors such as climbing represent the absence of a depression-like phenotype. It has recently been proposed that immobility in the FST is an adaptive learned response and reflects a switch from active to passive coping strategies (de Kloet and Molendijk, 2016). While we agree with de Kloet and Molendijk (2016) that immobility is adaptive we disagree on the interpretation of active versus passive coping strategies. Instead we believe that escape-directed behaviors are driven by anxiety. We argue this perspective on the basis of comorbidity, gene targeting, and pharmacological studies.

Depression rarely strikes alone. In clinical populations, depression is highly comorbid with anxiety disorders, with estimates as high as 80% (Lamers *et al*, 2011). Despite the high degree of comorbidity between depression and anxiety in clinical populations there are only a few studies examining individual differences in comorbidity between depression- and anxiety-like behaviors in rodents. One study found no relationship between performance on the FST and the elevated plus maze

(EPM) (Ho *et al*, 2002), whereas another study reported an inverse relationship between performance on the FST and the EPM. In other words, animals that were high on measures of depression-like behaviors were low on measures of anxiety-like behaviors (Estanislau *et al*, 2011). More recently, we conducted a study looking at individual differences in depression- and anxiety-like behaviors where our results are more consistent with Estanislau and colleagues (2011). The inverse relationship between depression- and anxiety-like behaviors is surprising as it is diametrically opposed to human clinical populations. There are two possible explanations for the discrepancies between human and animal research: either, the underlying mechanisms driving depression and anxiety are distinct in humans and rodents, or we are misinterpreting animal behavior. We believe the underlying mechanisms are conserved and therefore it is more likely due to interpretation error.

While comorbidity is the norm within clinical populations, much less is known about comorbidity in standard lab rodents. Different methodologies can be employed in the search for parallels between human comorbidity and animal models including gene knockouts (KO), selective breeding, and RNA interference. Although there are numerous animal models at one's disposal, we address two KO models: the serotonin transporter (5-HTT) KO and the serotonin 1A receptor (5-HT_{1A}) KO. These KO lines were chosen because the 5-HT system is implicated in both depression and anxiety disorders in clinical populations and that the most commonly used drugs to treat these disorders target the 5-HT system.

The precise mechanisms underlying the therapeutic effects of SSRIs have yet to be elucidated, however, it is known that SSRIs increase the amount of 5-HT

available at the synaptic cleft through blocking the re-uptake of 5-HT at 5-HTT sites. Holmes and colleagues (2003) have done extensive work characterizing the phenotype of 5-HTT KO mice and demonstrated that they exhibit increased anxiety-like behaviors in the EPM, open field, and light-dark box, spend more time immobile in the FST, are more passive and less aggressive in the resident-intruder paradigm, but are also less active overall as measured by baseline activity in their homecage (see Holmes *et al*, 2003). Considering that 5-HTT KO exhibit increased anxiety-like behaviors across a host of behavioral tests and spend more time immobile in the FST one could argue that knocking out 5-HTT generates an animal model of comorbid depression- and anxiety-like behavior. The fact that this KO model is generally less active is important to note because anxiety-like phenotypes are often based on activity levels, such as reduced exploratory activity in the open field and exploration of the EPM. As such, the general blunting of activity in 5-HTT KOs raises the question: can an overall reduction in activity account for the increased immobility observed in the FST as well as the anxiety-like phenotype? Given this caveat, researchers using KO models should pay close attention to overall activity levels to rule out this possibility.

In their work with the 5-HTT KOs, Holmes *et al.,* (2003) found that the anxiogenic phenotype is mediated through 5-HT_{1A} receptors. As with the 5-HTT gene, the 5-HT_{1A} receptor has been implicated in mood and anxiety in humans. 5-HT_{1A} receptor KO mice exhibit increased anxiety-like behaviors (Klemenhagen *et al*, 2006) as well as increased escape-directed behaviors in the FST (Freeman-Daniels *et al*, 2011), making 5-HT_{1A} KOs similar to 5-HTT KOs in respect to anxiety-like

behaviors but opposite in respect depression-like behaviors in the FST. Both the 5-HTT and the 5-HT_{1A} receptor are implicated in depression and anxiety in humans, yet KO mouse lines exhibit different behavioral phenotypes. To explain these differences, we maintain that the 'depression-like' phenotype associated with 5-HTT KO is the result of an overall blunting of activity. In respect to the 5-HT_{1A} KO, we argue that the persistance of escape-directed behavior in the FST does not represent an antidepressant-like response, but is instead caused by the anxiogenic effect of knocking out 5-HT_{1A} receptors.

In addition to exhibiting an anxiety-like phenotype, 5-HT_{1A} KOs also exhibit deficits in hippocampal-dependent learning tasks including contextual fear conditioning (Klemenhagen *et al*, 2006). There are a number of convincing lines of evidence which support the position that immobility is a learned response (reviewed in de Kloet *et al*, 2016). In brief, if memory consolidation following the habituation phase is blocked with the protein synthesis inhibitor anisomycin, animals do not become immobile on the test day 24h later (De Pablo *et al*, 1989). Memory consolidation in the FST appears to be mediated by glucocorticoid signaling because adrenalectomy has the same effect on immobility as anisomycin. Moreover, adrenalectomized rats that receive an injection of corticosterone 15-min after the habituation trial become immobile on test day (Veldhuis *et al*, 1985).

The claim that the FST measures depression-like behaviors is based on evidence from preclinical trials that antidepressant compounds reduce immobility. It is consistently shown that an acute injection of a putative antidepressant reduces immobility in the FST (see de Kloet and Molendijk, 2016; Van der Meersch-Mougeot

et al., 1993). The fact that acute exposure to an antidepressant can produce an antidepressant-like response in rodents has been criticized because antidepressants require weeks of chronic use in clinical populations. Moreover, many patients do not respond to the first antidepressant prescribed and many patients are deemed treatment-resistant because they do not respond to two or more courses of antidepressant treatment. The issues of delayed onset and questionable efficacy are acknowledged in some animal behavior studies, however, the fact that antidepressants are associated with the induction or exacerbation of anxiety, particularly during the acute stage of treatment, has not been acknowledged.

The fact that antidepressants induce or exacerbate anxiety in clinical populations suggests that antidepressants would also have anxiogenic properties in rodents. This is particularly important given that protocols designed to assess antidepressants in rodents focus on acute exposure to the drug. Consistent with our anxiogenic hypothesis, Silva and colleagues (1999) demonstrated that an acute injection of fluoxetine induces anxiety-like behaviors in rodents. Interestingly, chronic exposure to fluoxetine had sustained anxiogenic properties (Silva *et al*, 1999). The lack of anxiolytic properties associated with fluoxetine is surprising given that SSRIs are the first line treatment for anxiety disorders.

There is evidence from both clinical reports and animal studies demonstrating that antidepressants induce anxiety. Therefore, we must consider whether the anxiogenic effects of antidepressants are being misinterpreted as antidepressant properties. If reduced immobility and increased escape-directed behaviors are driven by anxiety, it follows that anxiogenic compounds would have

an anti-immobility effect in the FST. Nishimura and colleagues (1989) administered either beta-carboline-3-carboxylic acid ethyl ester (β-CCE; an inverse agonist at the benzodiazepine receptor site) or diazepam (an anxiolytic) to rats and tested their performance on the FST. They reported increased climbing and escape-directed behaviors in animals treated with β-CCE, whereas rats treated with diazepam spent significantly more time immobile (Nishimura *et al*, 1989). Likewise, the alpha-2-adrenergic receptor antagonist yohimbine, which exerts its anxiogenic properties through antagonism of alpha-2-receptors, has been shown to potentiate the anti-immobility effects of fluoxetine (Dhir and Kulkarni, 2007). Furthermore, benzodiazepines have also been shown to reverse the anti-immobility effects of antidepressants (Van der Meersch-Mougeot *et al*, 1993). Interestingly the reversal of anti-immobility is specific to antidepressants because benzodiazepines do not affect caffeine-induced activity in the FST. As such, we believe that the anxiolytic is attenuating the anxiogenic effect of acute exposure to antidepressants. Within the clinical sphere, some physicians and psychiatrists prescribe an anxiolytic for the first weeks when commencing antidepressant treatment in order to manage the anxiety associated with the treatment and maintain adherence. One possible side effect of anxiolytics is sedation, thus one could argue that the decreased activity in the diazepam condition is driven by the sedative effect of the drug. Although possible, this is unlikely given that anxiolytics promote exploratory activity in the EPM. Therefore, any sedative effects associated with the anxiolytic likely do not account for the increase in immobility in the FST. In summary, there is converging evidence from pharmacological studies demonstrating that antidepressants can

induce anxiety in both human and animal models. We argue that the antidepressant-induced anxiety is the driving force behind the increased escape-directed behaviors seen in the FST.

Conclusion

The FST is a widely used behavioral assay for depression-like behavior yet what immobility and escape-directed behaviors mean exactly remains unclear. Given the ubiquitous use of the FST to assess mood in gene KOs and other animal models of mood, it is necessary to have a clear understanding of what the test represents and what the behaviors actually mean. Drawing upon comorbidity, gene KOs and pharmacological studies from both animal models and clinical samples we propose an alternative interpretation of the FST. To further our understanding of the FST, future studies can continue to examine comorbidity between depression- and anxiety-like behaviors in healthy rodents. The study of individual differences as more of a top-down approach to understanding psychopathology will compliment what is learned from bottom-up approaches including gene KOs. While genetic models of mood and anxiety disorders provide insight into molecular pathways of a particular state, one must pay attention to confounding effects that can make inferring mood states more difficult. It is also important to focus on sex differences in mood-related behaviors. There are sex differences in the prevalence of depression and anxiety disorders in humans, which means future animal studies should include both male and female subjects. Finally, we believe that the

understanding and interpretation of animal behavior would be improved by looking to clinical research. Currently there is a resurgence of interest in the therapeutic benefits of compounds such as lysergic acid diethylamide and psilocybin. These compounds were not developed for antidepressant or anxiolytic purposes but have therapeutic benefits, most likely mediated through their high affinity for 5-HT receptors. It will be interesting to see how these drugs affect performance on the FST and other measures of anxiety-like behavior. When designing experiments with these drugs it will be important to look to human clinical trials for the time course. For example, it will be important to administer the drug at least 2-3 days prior to behavior testing. In moving forward, it is important that the methodology used in animal studies mirrors human clinical trials as closely as possible. Utilizing similar methodologies, in respect to dosages and time course in particular will promote a better understand of the relationship between performance on the FST and anxiety-like behaviors.

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