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Inconvenient truths and the usefulness of identifying unknown unknowns

We studied how the sex of human experimenters affected mouse behaviors and brain functions under normal conditions and in the context of ketamine administration. Identifying such unknown unknowns was critical to understanding how, specifically and quantitatively, they affected experimental outcomes, which led to fresh insight into ketamine's mechanism as an antidepressant drug.

This is a summary of:
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The question

Our laboratory has a focus on understanding the mechanism of action of antidepressant drugs. The most exciting finding in depression research in recent memory is that the anaesthetic ketamine, when administered at sub-anaesthetic doses, can produce a rapid and persistent antidepressant effect[1]. In mice, ketamine administration has been shown to produce antidepressant-like effects similar to those of other antidepressant drugs.

Our lab was having difficulty replicating this effect that we had previously shown in mice[2]. Our earlier findings were achieved with a male experimenter administering ketamine, whereas in these seemingly identical non-replicating studies a female experimenter was administering ketamine. Being familiar with earlier work identifying that the sex of the human experimenter can affect stress and behavioural responses in rodents[3], we began to ponder whether our replication issue was related to the sex of the human experimenter, but frankly, did not consider this to be likely.

The solution

To systematically rule out this possibility, we randomized administration of ketamine or vehicle to mice by either a male or female experimenter. Subsequently, a third experimenter, who was blind to the experimental groups, tested these mice using an antidepressant-sensitive behavioural assay, the forced swim test (FST). To our surprise, we found that only mice given ketamine by the male experimenter showed the expected antidepressant-like behavioural change on the FST. To be certain, we repeated the identical experiment multiple times with different male and female experimenters, and obtained the same result each time. The experiment was then repeated at a different university, which had made the same observation years earlier but had not systematically tested it, with the same result. We also found that the observations extended to other ketamine-sensitive antidepressant assays (Fig. 1a).

We then reasoned that the mechanism driving this effect could provide important insight into ketamine's mechanism as an antidepressant drug. First, we established that mice show an aversion to the scent of male experimenters and a preference for the scent of female experimenters (Fig. 1b), as well as an increase in stress susceptibility only when handled by male experimenters.

We hypothesized that these acute stress effects could interact with the antidepressant actions of ketamine. Corticotropin-releasing factor (CRF) is a primary mediator of stress responses, and we found that neurons located in the entorhinal cortex that express this neuropeptide were activated in response to male scent. We modulated the responses of mice to ketamine by increasing or decreasing CRF activity and found that activation of CRF neurons projecting from the entorhinal cortex to the hippocampus acted synergistically with ketamine (Fig. 1c). We conclude that exposure to the scent of male experimenters prior to ketamine administration was sufficient to activate these hippocampus-projecting entorhinal cortex CRF neurons, and that without it present the necessary synergy to produce the effect was absent.

The implications

Our observation is likely to be the tip of an iceberg. There are undoubtedly many unknown unknowns, perhaps of equal importance to the known knowns, that are influencing experimental outcomes[4]. An inconvenient truth is that the inability to replicate experimental results between and sometimes within laboratories may be due to unrecognized experimental variables that are not controlled. Whereas the known knowns can be accounted for, unknown unknowns are also leading to non-replicating results.

Many other factors may affect behavioral results or potentially interact with the sex of the experimenter to influence experimental outcomes[4,5]. These factors could include the use of ventilated or open-air cages and in-house bred or commercially supplied (and shipped) rodents; the strain and substrain of the animals; the circadian cycle phase during testing; unique handling procedures; and the stress level, diet, and hormonal status of experimenters and/or animals. These factors may explain why some laboratories find antidepressant effects following ketamine administration by female experimenters.

We recommend reporting the sex of the experimenter in the methods section of papers that include animal experiments and controlling for this variable when possible. Identification of further unrecognized variables should be supported and encouraged. The discovery of these interactions could lead to interesting new treatment approaches, such as the administration of ketamine combined with CRF receptor agonists as a treatment approach for depression.

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EXPERT OPINION

"There are several strengths to the study, including its use of several behavioral assays and stress procedures, such as chronic social defeat stress, and the fact that certain findings of differences between male vs. female experimenters were replicated at two different sites. I find the information on an EC–HP circuit which is differentially affected by male vs. female human scent especially compelling." **Eric Nestler, Icahn School of Medicine at Mount Sinai, New York, NY, USA.**

FIGURE

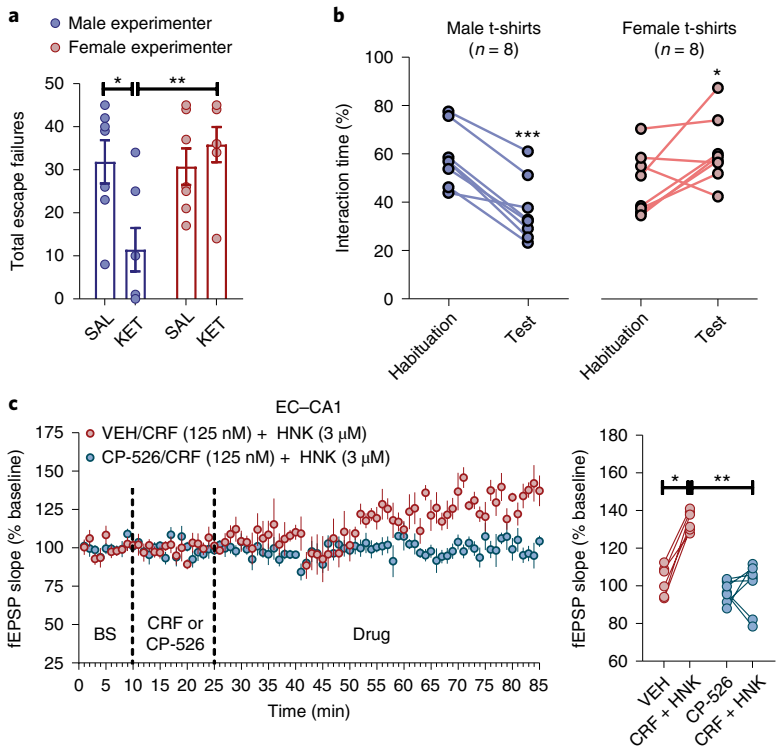

Fig. 1 | Sex of the human experimenter modulates antidepressant effects of ketamine through the CRF system. a, A single administration of ketamine by a male experimenter, but not a female experimenter, reversed learned helplessness behaviors in mice that were rendered helpless from a stress exposure. **b**, Mice showed avoidance of male-worn t-shirts and preference for female-worn t-shirts. **c**, The in vitro combination of CRF and ketamine's biologically active metabolite (*2R,6R*)-hydroxynorketamine (HNK) increased field excitatory postsynaptic potential (fEPSP) responses, a measure of synaptic strength, in the entorhinal cortex–hippocampal CA1 pathway; this increase was blocked by the CRF receptor 1 antagonist CP-526. © 2022, Georgiou, P. et al.

BEHIND THE PAPER

Prior to this project my primary experimental focus was not ketamine pharmacology. My main field of interest is neuroendocrinology, but it was not in my plans to investigate the differential effects of experimenters' sex on mouse behaviours. I, a female, noticed the lack of a typical ketamine behavioural response when I was filling in for a male postdoc. We first decided to test possible effects of sex of the experimenter mainly out of curiosity. Following our early results my PI initially discouraged me from extensively pursuing the characterization, but I wanted to know the mechanistic answer and help others in science facing replicability issues. Some people may have chuckled and questioned my scientific approach when asking to swab their skin or for donation of their clothing, but seeing the completed project I have the sense of satisfaction for making an important though unexpected scientific contribution. **P.G.**

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FROM THE EDITOR

"I found this paper intriguing because it not only provides a novel mechanism involved in the antidepressant-like effect of ketamine in mice, but also shows how this mechanism is engaged by an unexpected environmental factor — in this case, exposure to the scent of a male human. As such, it may have implications for the replicability of ketamine studies in mice." **Leonie Welberg, Senior Editor, *Nature Neuroscience*.**