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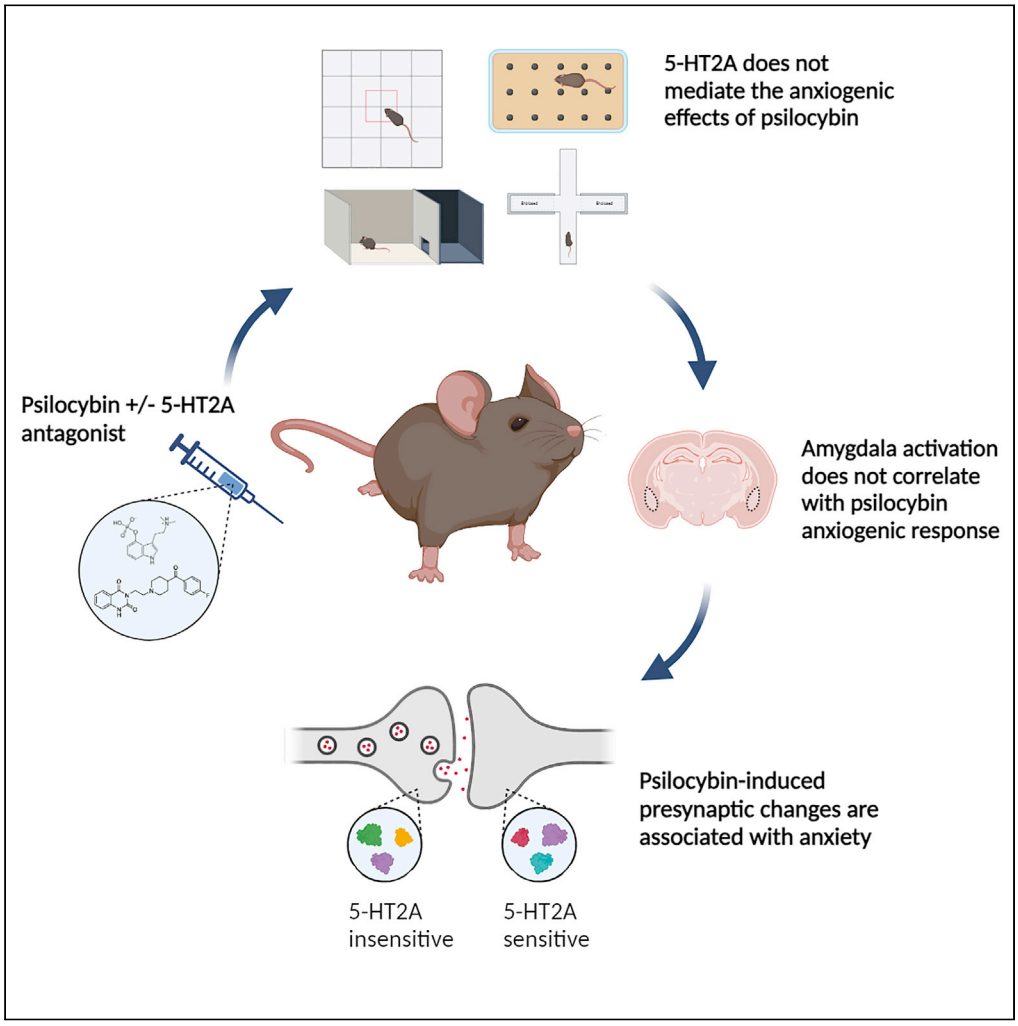

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Highlights

Psilocybin induces acute anxiety and neuronal activation in amygdala

5HT2a antagonist, ketanserin, does not attenuate psilocybin-induced anxiety

Psilocybin induces acute changes in protein phosphorylation levels in amygdala

Psilocybin induces protein phosphorylation changes in both presynaptic and postsynapse

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Psilocybin induces acute anxiety and changes in amygdalar phosphopeptides independently from the 5-HT2A receptor

Ram Harari,[1] Ipsita Chatterjee,[1,2] Dmitriy Getselter,[1] and Evan Elliott[1,3,*]

SUMMARY

Psilocybin, and its metabolite psilocin, induces psychedelic effects through activation of the 5-HT2A receptor. Psilocybin has been proposed as a treatment for depression and anxiety but sometimes induces anxiety in humans. An understanding of mechanisms underlying the anxiety response will help to better develop therapeutic prospects of psychedelics. In the current study, psilocybin induced an acute increase in anxiety in behavioral paradigms in mice. Importantly, pharmacological blocking of the 5-HT2A receptor attenuates psilocybin-induced head twitch response, a behavioral proxy for the psychedelic response, but does not rescue psilocybin's effect on anxiety-related behavior. Phosphopeptide analysis in the amygdala uncovered signal transduction pathways that are dependent or independent of the 5-HT2A receptor. Furthermore, presynaptic proteins are specifically involved in psilocybin-induced acute anxiety. These insights into how psilocybin may induce short-term anxiety are important for understanding how psilocybin may best be used in the clinical framework.

INTRODUCTION

Psilocybin has emerged as a major research interest, partly due to its potential as a treatment for several neuropsychological disorders, in particular anxiety and depression.[1–4] Psilocybin is a tryptamine alkaloid found in some species of mushrooms often called "magic mushrooms" and has a role in the psychedelic and hallucinogenic effects that these mushrooms induce.[5,6] The complete mechanisms of action of psilocybin are not fully understood.[7] Psilocybin's main psychedelic and hallucinogenic effects are through modulation of the serotonergic (5-HT) systems,[8–10] which is mediated by its metabolite psilocin, a non-selective serotonin 5-HT2A receptor agonist.[11,12] Previous studies in humans and mice have demonstrated that ketanserin, a 5-HT2A receptor antagonist, attenuate psilocybin's psychedelic and hallucinogenic effect.[10,13–15]

Rodent model studies have reported that exposure to psilocybin influences a variety of behaviors related to memory, learning, depression, and anxiety.[14,16–19] In addition, studies in mice, rats, and pigs found that psilocybin exposure alters neuron functional connectivity, structural remodeling, and gene expression within various brain regions.[14,17,20,21] Initial clinical studies have suggested the therapeutic efficiency of psilocybin in treating several neuropsychiatric disorders e.g., obsessive-compulsive disorder, addiction, depression, and anxiety.[22–25] Recent reports in human patients showed preferred or similar efficiency in antidepressant effects of psilocybin compared to escitalopram, a selective serotonin-reuptake inhibitor (SSRI).[26–29] In addition, a parallel study showed that psilocybin's antidepressant effect was associated with increased activation in various brain networks, while escitalopram did not have this effect.[30] These studies indicate that the therapeutic activity of psilocybin may be related to a different neuronal mechanism of action from that of the SSRI.

Interestingly, while psilocybin has been proposed as a treatment for depression and anxiety related disorders, it also has been reported that psilocybin sometimes induces acute anxiety, particularly in contexts related to distortion in self-perception and subject-object boundaries, mostly described as "dread of ego-dissolution" or the "fear of losing the sense of selfhood and the boundaries of self".[28,31] These adverse events in some cases have led to the need for psychological, psychiatric, or medical support.[32] Partly to mitigate adverse events related to anxiety symptoms, psilocybin-assisted psychological therapy has been employed as an integral component in psilocybin clinical studies, although the cost-effectiveness of this therapy is not clear.

The amygdala is part of the limbic system, which has a known role in the regulation of many emotional processes,[33] and is implicated in the pathophysiology of depression and anxiety.[33–36] Recent evidence indicates that the antidepressant effect of psilocybin treatment is associated with increased response in the amygdala.[37] In addition, a study on treatment-resistant depression patients also showed that psilocybin treatment influenced changes in amygdala functional connectivity during fearful face processing.[22] A more exact understanding of the role of the amygdala and underlying molecular pathways in psilocybin-induced anxiety can help to design future psilocybin-based treatments.

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The current study aims to understand how psilocybin affects acute anxiety and to determine the underlying cellular and molecular mechanisms. We have discovered that psilocybin induces acute anxiety in several anxiety-related tests in mice in parallel with an increase in neuronal activity in stress-related regions in the brain. We further determined that ketanserin, a 5-HT2A inhibitor, cannot attenuate the anxiety behavior although it can partly attenuate the increase in neuronal activity. Finally, we determined that psilocybin can induce acute changes in protein phosphorylation in the amygdala and that a significant proportion of these changes cannot be attenuated by ketanserin. These findings will contribute to our understanding of the mechanisms of psilocybin-induced acute anxiety.

RESULTS

Psilocybin increases acute anxiety-related behavior and neuronal activity in a dose dependent manner

The effect of psilocybin on acute anxiety-related behaviors was determined in a dose-dependent manner. A schematic representation of the experimental timeline is presented in Figure 1A. In the open field, 5 mg/kg psilocybin induced a significant decrease in time in center, as well as in % distance moved in center, compared with all other experimental groups (Figures 1B–1D and S1). In the dark-light test, all doses of psilocybin induced a significant decrease in time spent in light zone compared to control (Figure 1E). In the elevated plus maze, 3 mg/kg and 5 mg/kg psilocybin produced significant decrease in time spent in open arms and in % distance moved in open arms compared to 1 mg/kg and control (Figures 1F–1G and S1). These findings in all the anxiety-related behavior tests suggest that psilocybin has a role in acute anxiety-related behavior, while the dark-light test was able to detect anxiety-like behavior at even the smallest tested dose. In the marble burying test, 3 mg/kg and 5 mg/kg psilocybin produced a significant decrease in marbles buried compared with 1 mg/kg and control (Figure 1H). This finding is challenging to interpret since decreased marble burying is often considered indicative of less anxiety. However, several studies have suggested this test does not determine anxiety behaviors, but rather repetitive or perseverative behaviors,[38] and some mouse models with anxiety display decreased marbles buried.[39]

Immunohistochemistry staining for c-Fos expression in two stress-responsive brain regions, the basolateral amygdala and dentate gyrus, was performed to assess psilocybin-induced neuronal activity. Psilocybin induced a significant increase in c-Fos positive cells in the basolateral amygdala. 3 mg/kg and 5 mg/kg psilocybin induced an increase in c-Fos positive cells compared with 1 mg/kg psilocybin and control (Figures 2A and 2B). In addition, psilocybin induced a significant increase in c-Fos positive cells in the dentate gyrus of the hippocampus in the 3 mg/kg group, but there were no significant changes in the 5 mg/kg psilocybin group (Figures 2C and 2D). In general, psilocybin induced more pronounced increases in c-Fos positive cells in the basolateral amygdala, compared to the dentate gyrus.

Blocking of 5-HT2A receptor by ketanserin did not rescue psilocybin effect on acute anxiety-related behaviors

To decipher the role of serotonin 5-HT2A receptor in psilocybin's anxiety-related behavioral effects, we injected ketanserin (i.p.), a 5-HT2A receptor antagonist, which is known to attenuate psilocybin's psychedelic and hallucinogenic effects in humans and mice,[10,13,15] followed by injection (i.p.) of 5 mg/kg psilocybin (Figure 3A). First, to validate that ketanserin produces its desired effects, we used the head twitch response paradigm.[13] Psychedelic effects cannot be directly measured in mice, and head twitching is most often used as a proxy for psychedelic effects in mice. It has previously been reported that ketanserin can attenuate the head twitch response.[13] Preinjection of mice with ketanserin attenuated the psilocybin-induced increase in head twitches (Figure 3B). In other words, psilocybin-PBS group had significantly more head twitches than all other experimental groups, including the psilocybin-ketanserin group. In the open field, both psilocybin-kentanserin and psilocybin-PBS groups spend significantly less time in center than the PBS-PBS or PBS-ketanserin groups. There was no difference between the psilocybin-PBS and psilocybin-kentanserin group (Figure 3C). Therefore, ketanserin did not attenuate the effect of psilocybin. These same findings were seen also in the dark-light and elevated plus maze tests (Figures 3D and 3E). In all these tests, ketanserin did not attenuate the psilocbyin-induced anxiety. In addition, ketanserin did not affect the decrease in marble burying behavior caused by psilocybin (Figure 3F). These results suggest a lack of a role for 5-HT2A receptor in psilocybin-induced acute anxiety.

Ketanserin rescues psilocybin's effect on c-Fos expression in the basolateral amygdala and paraventricular nucleus of the hypothalamus

To decipher the role of serotonin 5-HT2A receptor in psilocybin-induced neuronal activation, we performed c-Fos immunohistochemistry after treatment of animals with psilocybin and/or ketanserin. The PBS-psilocybin group displayed increased c-Fos positive cells compared to all other experimental groups. Ketanserin-psilocybin group displayed significantly lower c-Fos positive cells than the PBS-psilocybin group, but not significantly different than the PBS-PBS or PBS-ketanserin groups. Therefore, ketanserin attenuated psilocybin-induced increase in c-Fos positive cells in the basolateral amygdala (Figures 4A and 4B). Considering that the hypothalamus, and particularly the paraventricular nucleus (PVN), also affects anxiety-related behaviors, we determined the effects of psilocybin and ketanserin in the PVN. We found a similar pattern of c-Fos activity among the experimental groups in the PVN as we saw in the basolateral amygdala (Figures 4C and 4D). The ketanserin-psilocybin group displayed significantly less c-Fos staining than the PBS-psilocybin group, suggesting that c-Fos activation in the PVN is at least partially mediated through the 5-HT2A receptor. Since ketanserin alone had some positive effects on c-Fos activation, it is difficult to decipher if ketanserin completely blocked the effects of psilocybin. However, considering that there is no difference between ketanserin-PBS and ketanserin-psilocybin groups, we can determine that psilocybin has no significant effects on c-Fos levels in the PVN in the presence of ketanserin. Therefore, psilocybin-induced changes in c-Fos positive cells in basolateral amygdala and PVN are mediated through the 5-HT2A receptor.

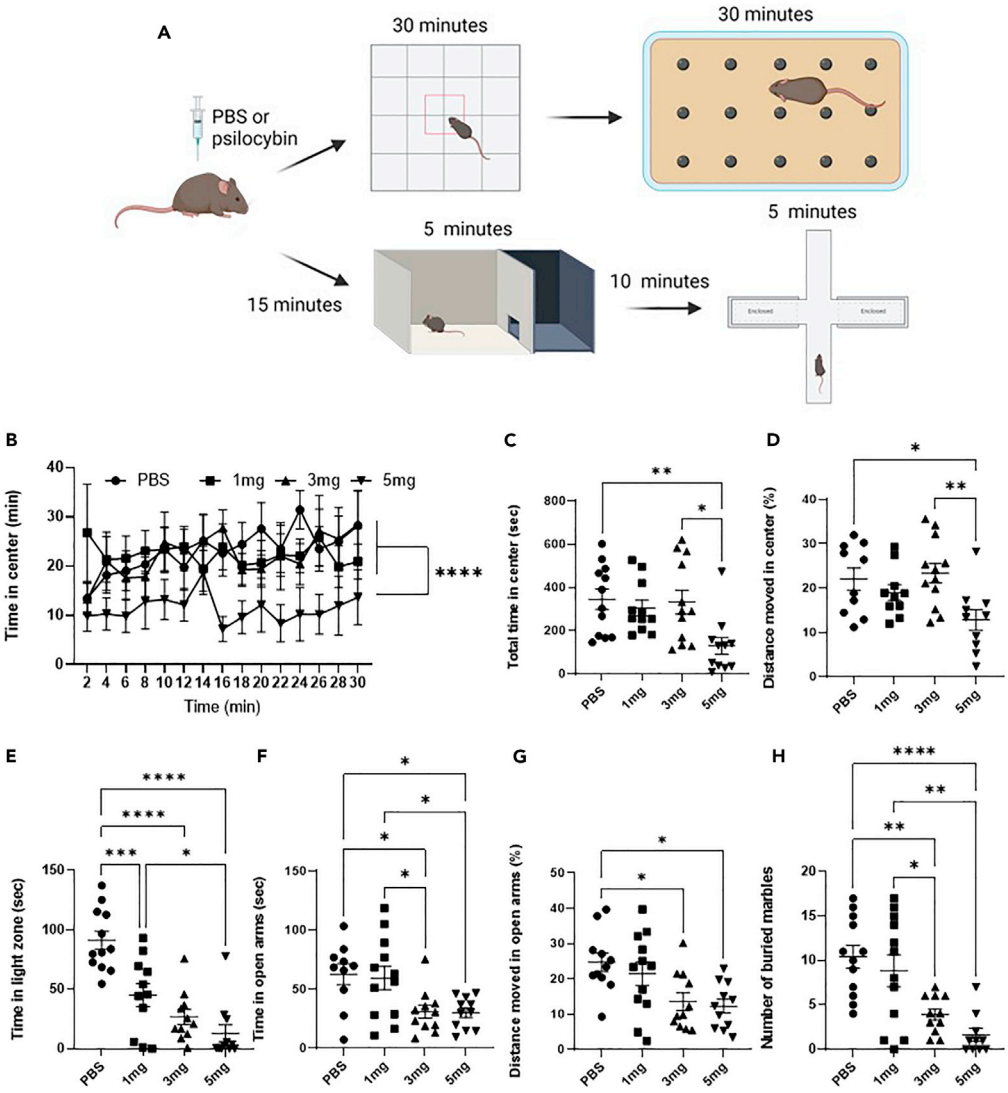

Figure 1. The effect of psilocybin on anxiety-related behavioral tests

(A) Schematic representation of experimental design, created with BioRender.com.

(B) Time spent in center of open field. The test was carried out for 30 min and data are time bins of every 2 min within the 30 min.

(C) Total time spent in the center of the open field test in 30 min.

(D) Percent total distance traveled in the center of the open field. This is calculated by (total distance traveled in center)/(total distance traveled in total arena).

(E) Total time spent in light zone of dark-light test in 5 min time period.

(F) Total time spent in open arms of elevated plus maze in 5 min time period.

(G) Percent distance traveled in the open arms of the elevated plus maze. This is calculated by (distance traveled in open arms)/(distance traveled in entire maze).

(H) Marbles buried within 30 min period in marble burying test. Values represent mean ± SEM, $n = 12$, */**/***/****$p < 0.05/0.01/0.001/0.0001$ by two-way ANOVA and one-way ANOVA, followed by Tukey's multiple comparisons test.

Phosphoprotein analysis in the amygdala

Acute behavioral effects of pharmacological agents are often due to immediate modulation of protein phosphorylation. Protein phosphorylation changes can occur within minutes of receptor activation and are therefore a primary mechanism for mediating immediate effects of ligands on behavior. Therefore, to gain insight into the molecular mechanism of psilocybin-induced anxiety, we performed mass spectrometry to detect levels of proteins and phosphorylated proteins in the entire amygdala of mice treated with psilocybin alone or treated with psilocybin and ketanserin (Figure 5A). Amygdala was dissected 15 min after treatment with psilocybin or vehicle. We identified 796 phosphopeptides that were differentially phosphorylated by psilocybin, of those 74 displayed increased phosphorylation and 722 displayed decreased phosphorylation (Table S1). In total, only three proteins were differentially abundant (total protein levels) between experimental groups. This indicates that psilocybin induces immediate changes in protein phosphorylation without changes in protein levels at 15 min after

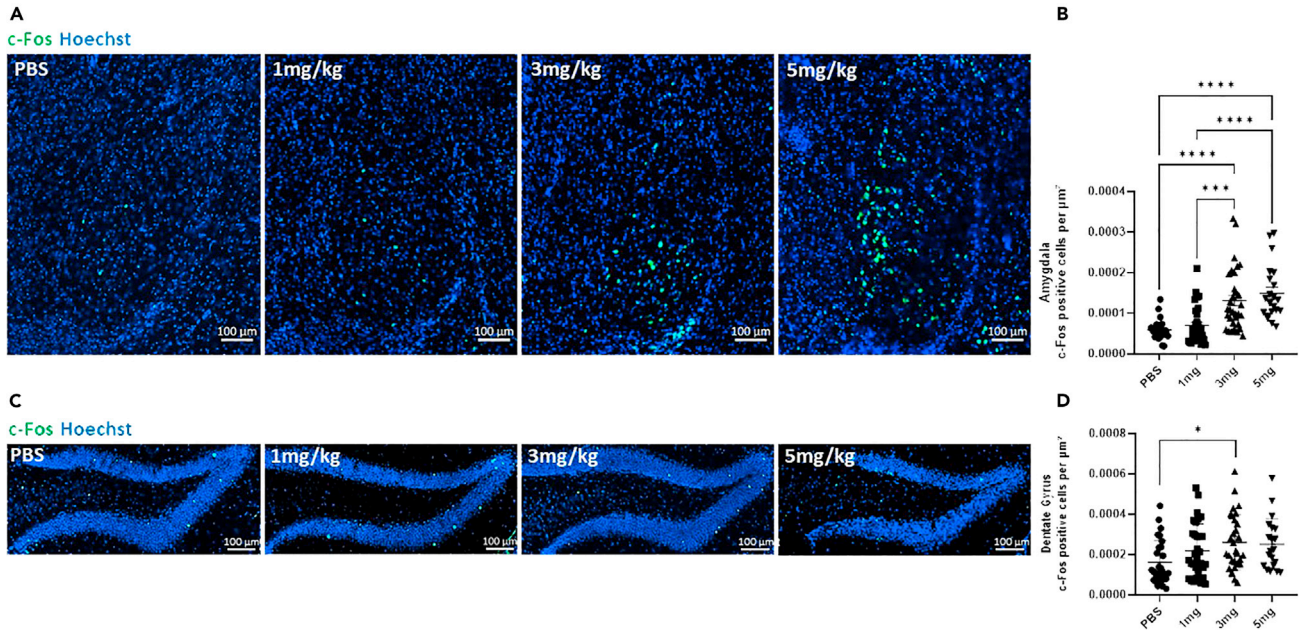

Figure 2. Effects of psilocybin on neuronal activation at the basolateral amygdala and dentate gyrus

(A) Representative immunostaining of central amygdala for c-Fos expressing cells (green) and Hoechst (blue) of 1 mg/kg, 3 mg/kg, and 5 mg/kg doses of psilocybin and PBS-control. Scale bar, 100 µm.

(B) Quantification of c-Fos expressing cells in the basolateral amygdala.

(C) Representative immunostaining of dentate gyrus for c-Fos expressing cells (green) and Hoechst (blue) of 1 mg/kg, 3 mg/kg, and 5 mg/kg doses of psilocybin and PBS-control. Scale bar, 100 µm.

(D) Quantification of c-Fos expressing cells in the dentate gyrus. Values represent mean ± SEM, basolateral amygdala $n = 21–32$ slices and dentate gyrus $n = 20–32$ slices, taken from 5 animals per group, */**/***/****$p < 0.05/0.01/0.001/0.0001$ by one-way ANOVA, followed by Tukey's multiple comparisons test.

treatment. Of the differentially abundant phosphopeptides, we identified 310 phosphopeptides that were significantly changed by psilocybin, but whose phosphorylation patterns were reversed by ketanserin (ketanserin-sensitive) (Figure 5B). In contrast, we found 486 phosphopeptides that were significantly changed by psilocybin, but were unaffected by ketanserin (ketanserin-insensitive) (Figure 5E). Therefore, these ketanserin-insensitive peptides may be regulated by pathways other than the 5-HT2A receptor and may be related to the acute anxiety-response. Ketanserin-sensitive proteins were enriched in the basal dendrite and synapse, particularly in the postsynaptic density (Figure 5C). In addition, there was an enrichment for proteins with the GKAP, CamkII, and SAPAP domains (Figure 5D). Of interest, ketanserin-insensitive phosphoproteins were enriched in the axon and presynapse (Figure 5F).

Protein-protein interaction analysis (PPI) was performed on both the ketanserin-sensitive and ketanserin-insensitive phosphopeptides to discover networks of interacting phosphoproteins that were dysregulated in each of the two experimental groups. In the ketanserin-sensitive group, PPI detected a module of proteins with the protein SYNGAP1 as a strong node with connections to multiple dysregulated phosphopeptides (Figure 6A). The module is highly enriched for postsynaptic proteins and modulators of chemical synaptic transmission (Figures 6B, 6C, and 6D). In the ketanserin-insensitive group, PPI detected a module of proteins found in the presynapse (Figures 6F and 6H), with SNAP25 and Syt1 as strong nodes in the module (Figure 6E). This module was highly enriched for SNARE binding proteins (Figure 6G), which are major presynaptic proteins modulating neurotransmitter release, and proteins regulation both neurotransmitter levels and secretion. Therefore, PPI validates specific presynaptic pathways regulated by psilocybin independent of the 5-HT2A receptor, while validating postsynaptic pathways regulated by psilocybin in a 5-HT2A-dependant manner.

DISCUSSION

We have tested psilocybin's effect on acute anxiety-related behavior in mice. In all behavioral tests, psilocybin significantly increases anxiety-related behaviors in a dose-dependent manner. The marble burying and open field test results were in concordance with other reports showing that psychedelic 5-HT2AR agonists, including psilocybin, can increase acute anxiety-related behavior in these specific tests.[18,19,40] However, previous studies did not report the effects of psilocybin in the light-dark and elevated plus maze tests. Therefore, these findings give further credence to the acute anxiogenic properties of psilocybin.

We have investigated the role of serotonin 5-HT2A receptor in psilocybin's behavioral effects and neuronal activation by pharmacological blocking of 5-HT2A receptor by ketanserin. We saw that ketanserin attenuates psilocybin head twitch response as shown in previous studies.[13,14] However, ketanserin did not attenuate psilocybin's effect on anxiety-related behavior. These results suggest a role for the 5-HT2A receptor in the hallucinatory effect of psilocybin but with little role in acute anxiety-related behavior. Several other studies have

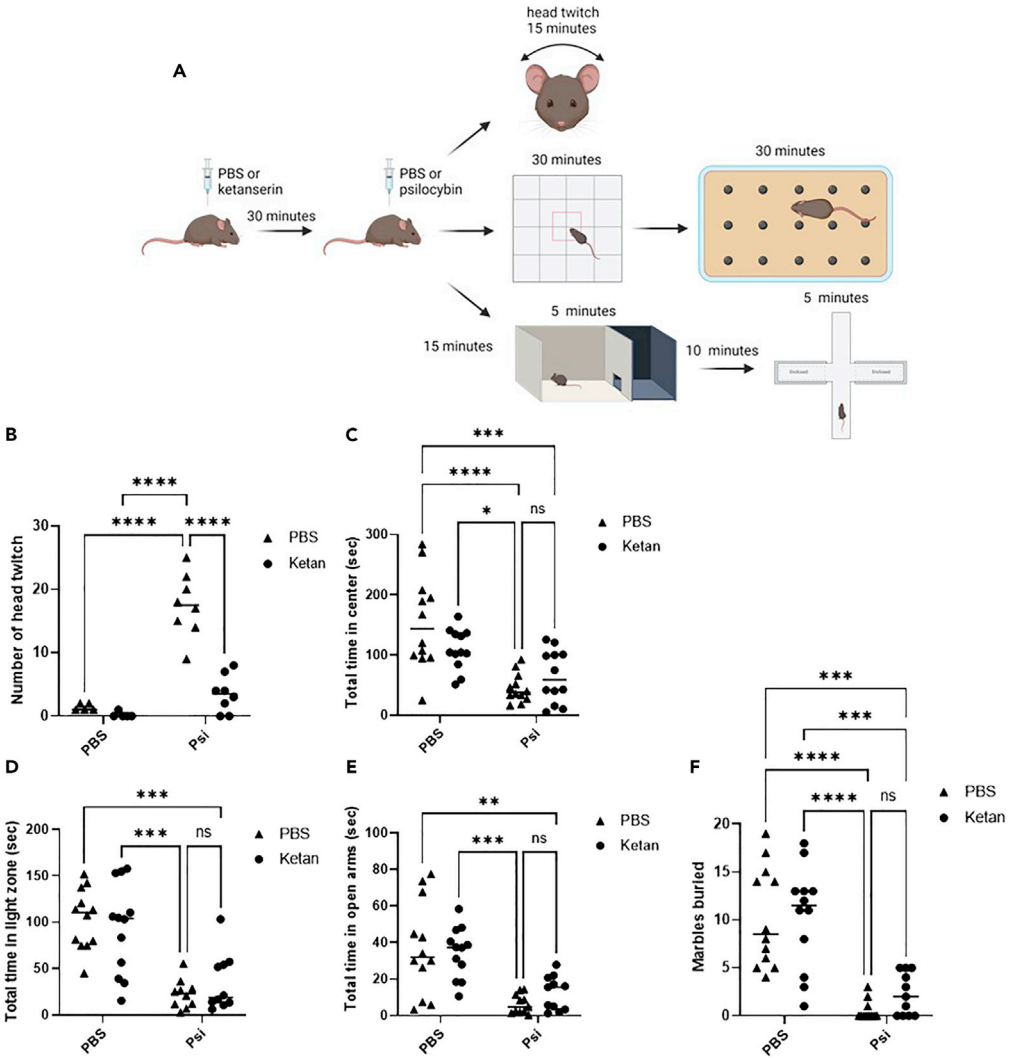

Figure 3. Ketanserin does not attenuate psilocybin effect on anxiety-related behavior

(A) Schematic representation of experimental design, created with BioRender.com.

(B) Number of head twitches in 15 min time period.

(C) Total time spent in the center of the open field test in 30 min.

(D) Total time spent in light zone of dark-light test in 5 min time period.

(E) Total time spent in open arms of elevated plus maze in 5 min time period.

(F) Marbles buried within 30 min period in marble burying test. Values represent mean ± SEM, $n = 12$, */**/***/****$p < 0.05/0.01/0.001/0.0001$ by two-way ANOVA, followed by Tukey's multiple comparisons test.

also reported that some of psilocybin's effects are not mediated through the 5-HT2A receptor. For example, a study in which mice were chronically stressed and treated with psilocybin, followed by ketanserin, reported that the anti-depressant effects of psilocybin could also not be attenuated by the 5-HT2A antagonist ketanserin.[41] However, it is not yet clear what the other pathways are through which psilocybin, and psilocin, may mediate behavioral effects. Of great interest, a recent study reported that psychedelics, including psilocybin, can bind TrkB, a receptor for BDNF (Brain Derived Neurotrophic Factor), and have neurotrophic and neuroplasticity effects through this interaction independently of serotonin 5-HT2A receptor activation.[42] While TrkB often has antidepressant properties, one study found that hyperactivation of TrkB is responsible for anxiety behavior in a zebrafish model of tuberous sclerosis complex,[43] which suggests TrkB may have anxiety promoting properties in some contexts. However, it is not clear if TrkB hyperactivity can have the same effect in mice or humans. Therefore, further studies are still necessary to uncover the exact receptors through which psilocybin has its effects on acute anxiety.

We found that psilocybin produced significant increases in c-Fos expression in the basolateral amygdala. These results emphasize that psilocybin has an acute effect on basolateral amygdala activation, which is a brain region known to mediate the perception of fear and anxiety-related behavior in mammals and humans.[17,33,35] However, ketanserin attenuated the psilocybin-induced increase in c-Fos expression in

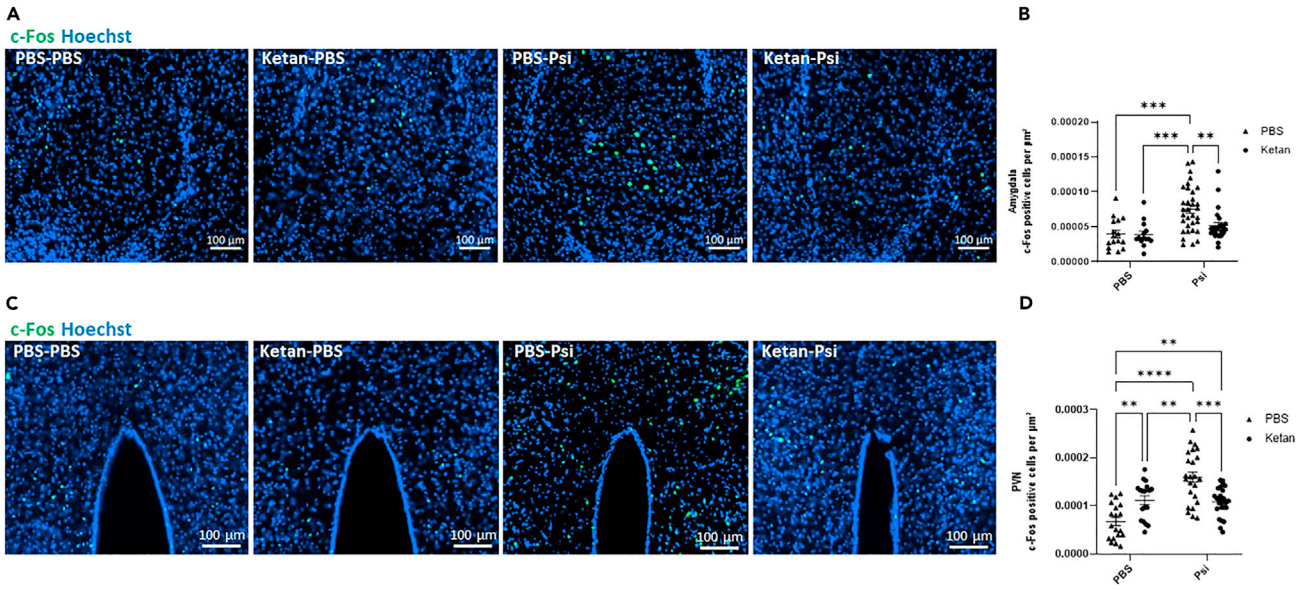

Figure 4. Ketanserin attenuates psilocybin's effect on neuronal activation at the basolateral amygdala and PVN

(A and C) Representative immunostaining for c-Fos expressing cells (green) and Hoechst (blue) in basolateral amygdala and PVN of PBS preinjected with PBS (PBS-PBS), PBS preinjected with ketanserin (Ketan-PBS), psilocybin 5 mg/kg dose preinjected with PBS (PBS-Psi), and psilocybin 5 mg/kg dose preinjected with ketanserin (Ketan-Psi). Scale bar, 100 µm.

(B and D) Quantification of c-Fos expressing cells in basolateral amygdala and PVN. Values represent mean ± SEM, basolateral amygdala n = 14–33 slices and PVN n = 18–25 slices taken from 5 animals per group */**/***/**** $p < 0.05/0.01/0.001/0.0001$ by two-way ANOVA, followed by Tukey's multiple comparisons test.

the basolateral amygdala. This result suggests that the acute anxiety-related behavior induced by psilocybin is not associated with the increased c-Fos activation in the basolateral amygdala.

While total c-Fos activation in the basolateral amygdala did not correlate with the acute anxiogenic response, it is still possible that the amygdala is playing an important role in the anxiogenic response. First, there are many cell types in the amygdala, and it is possible that only activation of a particular cell type is correlated with the anxiogenic response, but this cannot be detected at the level of total c-Fos activation. In addition, while c-Fos is a marker for intense neuronal activity, it does not relay information regarding more subtle changes in neuronal firing or synaptic plasticity.[44] Therefore, we performed a more in-depth analysis of molecular changes in the amygdala by looking at changes in peptide phosphorylation.

To gain insight into the molecular mechanism underlying the effects of psilocybin and the possible role of changes in the amygdala in the acute anxiogenesis, we performed phosphoprotein analysis of the amygdala shortly after exposure to psilocybin with or without ketanserin. Of great interest, we found that psilocybin induced changes in phosphorylation of both postsynaptic and presynaptic proteins. However, changes in postsynaptic proteins could be largely attenuated by ketanserin treatment, while presynaptic changes could not. This suggests that the presynaptic effects of psilocybin are not mediated by the 5-HT2A receptor. Since anxiety was also not mediated by the 5HT2A receptor, this further suggests that presynaptic changes are associated with anxiety. A previous study found that a single dose of psilocybin induced an increase in the presynaptic protein synaptic vesicle protein 2A (SV2A) in the pig brain.[21] One of the presynaptic proteins that was regulated by was SNAP25. A separate study found that genetic mutation of SNAP25 in mice induced increased anxiety.[45] An important continuation of the present study would be to perform electrophysiology recordings in amygdalar neurons after psilocybin stimulation to determine if there are specific differences in how psilocybin regulated presynaptic vs. postsynaptic potentials. Those electrophysiology techniques could verify specific roles for psilocybin in presynaptic functioning that are associated with behavior.[46] Another important future study would be to determine if there are specific phosphoproteins that directly mediate the effects of psilocybin on behavior. This could be deciphered by targeting a subset of phosphoproteins identified in the current studies using pharmacological tools followed by behavioral analysis. In total, our findings suggest that psilocybin-induced acute anxiety is associated with presynaptic changes in phosphoproteins, although not with changes in overall c-Fos activation in the amygdala.

Further research should also investigate the long-term consequences of acute anxiety during the psilocybin experience. In other words, does acute anxiety affect the long-term therapeutic effect of psilocybin? One human study found that challenging experiences during psilocybin treatment were negatively correlated to antidepressant effect.[47] However, a recent rodent study found that the transient increase of glucocorticoids, a stress hormone, was necessary for the long-term anti-anxiety effects of psilocybin.[48] Therefore, the exact role of acute anxiety in the long-term behavioral effects still needs to be elucidated.

In conclusion, our experiments demonstrate that psilocybin causes acute anxiety-related behavior effects in mice which is associated with molecular changes in the amygdala; however, not necessarily through interaction with the serotonin 5-HT2A receptor. It is possible that

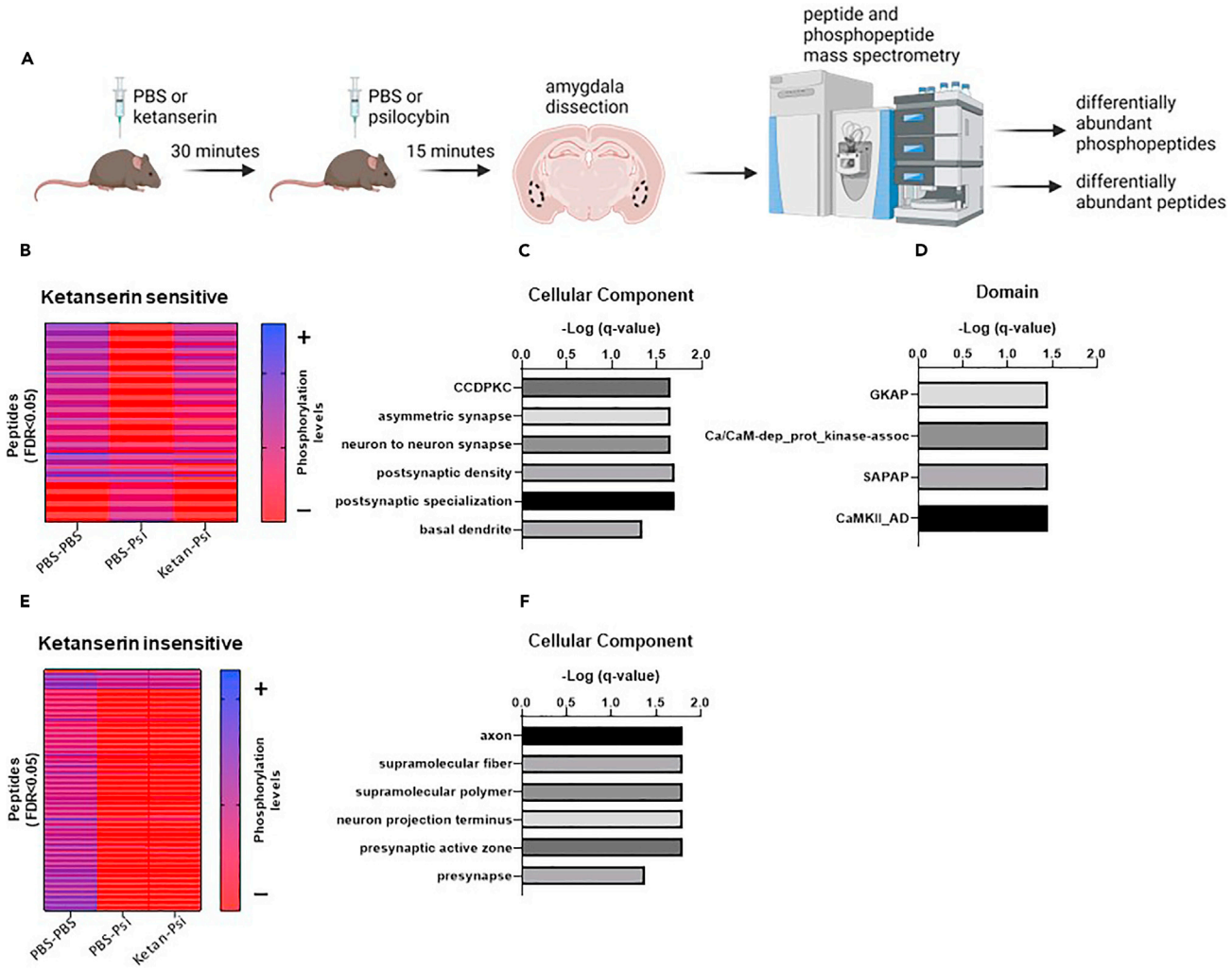

Figure 5. Psilocybin induces acute changes in levels of phosphopeptides in amygdala

(A) Schematic representation of experimental design, created with BioRender.com.

(B) Phosphopeptides that were significantly changed by psilocybin but reversed by ketanserin (ketanserin-sensitive).

(C and D) Gene Ontology (GO) enrichment analysis for ketanserin-sensitive phosphopeptides. GO categories with FDR<0.05 are shown.

(E) Phosphopeptides that were significantly changed by psilocybin but were unaffected by ketanserin (ketanserin-insensitive).

(F) GO enrichment analysis for ketanserin-insensitive phosphopeptides for cellular component. GO categories with FDR<0.05 are shown.

psilocybin is not only a 5-HT2A receptor agonist but may interact with other 5-HT receptor subtypes and/or additional systems to activate specific neurons to facilitate anxiety-related behavior.

Limitations of the study

There are two main limitations of this study. One limitation is that we do not have exact information on which cell type displayed psilocybin-induced neuronal activity. Information on cell type specificity (pyramidal neuron, interneuron, etc.) would give more valuable information into how psilocybin affects amygdala function. A second limitation of this study is that the phosphoproteomic analysis was performed on the whole amygdala, and not on a specific subregion. It is possible that subregion analysis would uncover more specific phosphoproteomic changes associated with behavioral changes.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- RESOURCE AVAILABILITY

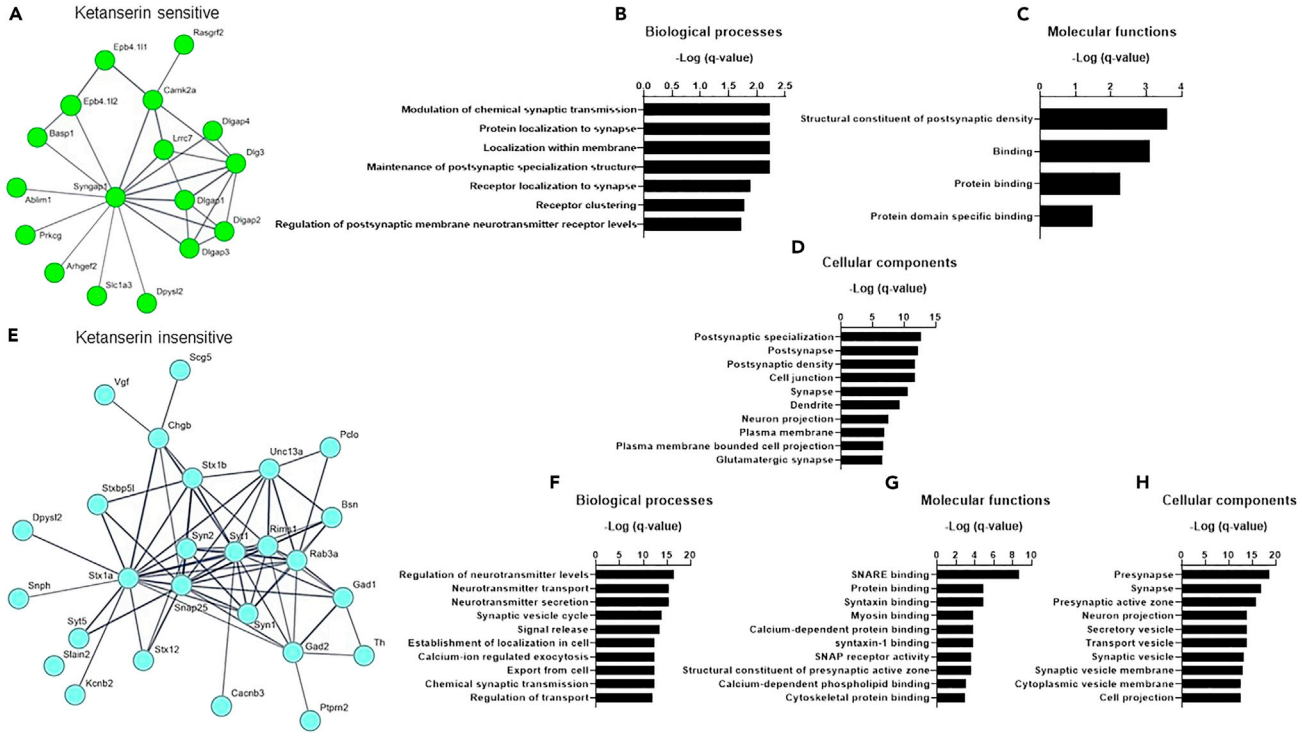

Figure 6. Protein-protein interaction network for phosphopeptides significantly changed by psilocybin treatment

(A and E) Ketanserin-sensitive (A) and ketanserin-insensitive (E) module of proteins clusters involved in common biological processes/molecular function/cellular components. Each circle represents a protein changed by the treatment. The thickness of lines represents the strength of data supporting a protein-protein interaction.

(B–D) Gene Ontology for the ketanserin-sensitive cluster of (B) biological processes, (C) molecular function, and (D) cellular components.

(F–H) Gene ontology for ketanserin-insensitive cluster of (F) biological processes, (G) molecular function, and (H) cellular components.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109686.

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AUTHOR CONTRIBUTIONS

R.H. performed molecular and behavioral experimentation and wrote the manuscript. I.C. performed analysis on behavioral experimentation. D.G. assisted in statistical analysis on behavioral experimentation and planning of experimentation. E.E. planned the study, supervised all aspects of the study and helped in drafting the manuscript. All authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Davis, A.K., Barrett, F.S., May, D.G., Cosimano, M.P., Sepeda, N.D., Johnson, M.W., Finan, P.H., and Griffiths, R.R. (2021). Effects of Psilocybin-Assisted Therapy on Major Depressive Disorder: A Randomized Clinical Trial. JAMA Psychiatr. *78*, 481–489. https://doi.org/10.1001/jamapsychiatry.2020.3285.
2. Patra, S. (2016). Return of the psychedelics: Psilocybin for treatment resistant depression. Asian J. Psychiatr. *24*, 51–52. https://doi.org/10.1016/j.ajp.2016.08.010.
3. Vargas, A.S., Luís, Â., Barroso, M., Gallardo, E., and Pereira, L. (2020). Psilocybin as a new approach to treat depression and anxiety in the context of life-threatening diseases-a systematic review and meta-analysis of clinical trials. Biomedicines *8*, 331. https://doi.org/10.3390/biomedicines8090331.
4. Goldberg, S.B., Pace, B.T., Nicholas, C.R., Raison, C.L., and Hutson, P.R. (2020). The experimental effects of psilocybin on symptoms of anxiety and depression: A meta-analysis. Psychiatry Res. *284*, 112749. https://doi.org/10.1016/j.psychres.2020.112749.
5. Geiger, H.A., Wurst, M.G., and Daniels, R.N. (2018). DARK Classics in Chemical Neuroscience: Psilocybin. ACS Chem. Neurosci. *9*, 2438–2447. https://doi.org/10.1021/acschemneuro.8b00186.
6. Passie, T., Seifert, J., Schneider, U., and Emrich, H.M. (2002). The pharmacology of psilocybin. Addict. Biol. *7*, 357–364. https://doi.org/10.1080/1355621021000005937.
7. Tylš, F., Páleníček, T., and Horáček, J. (2014). Psilocybin - Summary of knowledge and new perspectives. Eur. Neuropsychopharmacol. *24*, 342–356. https://doi.org/10.1016/j.euroneuro.2013.12.006.
8. De Gregorio, D., Enns, J.P., Nuñez, N.A., Posa, L., and Gobbi, G. (2018). D-Lysergic acid diethylamide, psilocybin, and other classic hallucinogens: Mechanism of action and potential therapeutic applications in mood disorders. In Progress in Brain Research (Elsevier B.V.), pp. 69–96. https://doi.org/10.1016/bs.pbr.2018.07.008.
9. López-Giménez, J.F., and González-Maeso, J. (2018). Hallucinogens and serotonin 5-HT2A receptor-mediated signaling pathways. Curr. Top. Behav. Neurosci. *36*, 45–73. https://doi.org/10.1007/7854_2017_478.
10. Vollenweider, F.X., Vollenweider-Scherpenhuyzen, M.F., Bäbler, A., Vogel, H., and Hell, D. (1998). Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. Neuroreport *9*, 3897–3902. https://doi.org/10.1097/00001756-199812010-00024.
11. Nichols, D.E. (2020). Psilocybin: from ancient magic to modern medicine. J. Antibiot. (Tokyo). *73*, 679–686. https://doi.org/10.1038/s41429-020-0311-8.
12. Winter, J.C., Rice, K.C., Amorosi, D.J., and Rabin, R.A. (2007). Psilocybin-induced stimulus control in the rat. Pharmacol. Biochem. Behav. *87*, 472–480. https://doi.org/10.1016/j.pbb.2007.06.003.
13. González-Maeso, J., Weisstaub, N.V., Zhou, M., Chan, P., Ivic, L., Ang, R., Lira, A., Bradley-Moore, M., Ge, Y., Zhou, Q., et al. (2007). Hallucinogens Recruit Specific Cortical 5-HT2A Receptor-Mediated Signaling Pathways to Affect Behavior. Neuron *53*, 439–452. https://doi.org/10.1016/j.neuron.2007.01.008.
14. Shao, L.X., Liao, C., Gregg, I., Davoudian, P.A., Savalia, N.K., Delagarza, K., and Kwan, A.C. (2021). Psilocybin induces rapid and persistent growth of dendritic spines in frontal cortex in vivo. Neuron *109*, 2535–2544.e4. https://doi.org/10.1016/j.neuron.2021.06.008.
15. Carter, O.L., Burr, D.C., Pettigrew, J.D., Wallis, G.M., Hasler, F., and Vollenweider, F.X. (2005). Using psilocybin to investigate the relationship between attention, working memory, and the serotonin 1A and 2A receptors. J. Cognit. Neurosci. *17*, 1497–1508. https://doi.org/10.1162/089892905774597191.
16. Hibicke, M., Landry, A.N., Kramer, H.M., Talman, Z.K., and Nichols, C.D. (2020). Psychedelics, but Not Ketamine, Produce Persistent Antidepressant-like Effects in a Rodent Experimental System for the Study of Depression. ACS Chem. Neurosci. *11*, 864–871. https://doi.org/10.1021/acschemneuro.9b00493.
17. Catlow, B.J., Song, S., Paredes, D.A., Kirstein, C.L., and Sanchez-Ramos, J. (2013). Effects of psilocybin on hippocampal neurogenesis and extinction of trace fear conditioning. Exp. Brain Res. *228*, 481–491. https://doi.org/10.1007/s00221-013-3579-0.
18. Matsushima, Y., Shirota, O., Kikura-Hanajiri, R., Goda, Y., and Eguchi, F. (2009). Effects of psilocybe argentipes on marble-burying behavior in mice. Biosci. Biotechnol. Biochem. *73*, 1866–1868. https://doi.org/10.1271/bbb.90095.
19. Odland, A.U., Kristensen, J.L., and Andreasen, J.T. (2021). Investigating the role of 5-HT2A and 5-HT2C receptor activation in the effects of psilocybin, DOI, and citalopram on marble burying in mice. Behav. Brain Res. *401*, 113093. https://doi.org/10.1016/j.bbr.2020.113093.
20. Martin, D.A., and Nichols, C.D. (2016). Psychedelics Recruit Multiple Cellular Types and Produce Complex Transcriptional Responses Within the Brain. EBioMedicine *11*, 262–277. https://doi.org/10.1016/j.ebiom.2016.08.049.
21. Raval, N.R., Johansen, A., Donovan, L.L., Ros, N.F., Ozenne, B., Hansen, H.D., and Knudsen, G.M. (2021). A single dose of psilocybin increases synaptic density and decreases 5-HT2A receptor density in the pig brain. Int. J. Mol. Sci. *22*, 835. https://doi.org/10.3390/ijms22020835.
22. Mertens, L.J., Wall, M.B., Roseman, L., Demetriou, L., Nutt, D.J., and Carhart-Harris, R.L. (2020). Therapeutic mechanisms of psilocybin: Changes in amygdala and prefrontal functional connectivity during emotional processing after psilocybin for treatment-resistant depression. J. Psychopharmacol. *34*, 167–180. https://doi.org/10.1177/0269881119895520.
23. Moreno, F.A., Wiegand, C.B., Taitano, E.K., and Delgado, P.L. (2006). Safety, tolerability, and efficacy of psilocybin in 9 patients with obsessive-compulsive disorder. J. Clin. Psychiatry *67*, 1735–1740. https://doi.org/10.4088/JCP.v67n1110.
24. Bogenschutz, M. (2015). Mood, craving, and self-efficacy in psilocybin-assisted treatment of alcoholism. Neuropsychopharmacology *40*.
25. ACTRN12619001225101 (2019). Psilocybin-assisted psychotherapy for the treatment of depression and anxiety associated with life-threatening illness. https://trialsearch.who.int/Trial2.aspx?TrialID=ACTRN12619001225101.
26. Carhart-Harris, R., Giribaldi, B., Watts, R., Baker-Jones, M., Murphy-Beiner, A., Murphy, R., Martell, J., Blemings, A., Erritzoe, D., and Nutt, D.J. (2021). Trial of Psilocybin versus Escitalopram for Depression. N. Engl. J. Med. *384*, 1402–1411. https://doi.org/10.1056/nejmoa2032994.
27. Nayak, S.M., Bari, B.A., Yaden, D.B., Spriggs, M.J., Rosas, F.E., Peill, J.M., Giribaldi, B., Erritzoe, D., Nutt, D.J., and Carhart-Harris, R. (2023). A Bayesian Reanalysis of a Trial of Psilocybin Versus Escitalopram for Depression. Psychedelic Med. *1*, 18–26. https://doi.org/10.1089/psymed.2022.0002.
28. Barba, T., Buehler, S., Kettner, H., Radu, C., Cunha, B.G., Nutt, D.J., Erritzoe, D., Roseman, L., and Carhart-Harris, R. (2022). Effects of psilocybin versus escitalopram on rumination and thought suppression in depression. BJPsych Open *8*, e163. https://doi.org/10.1192/bjo.2022.565.
29. Weiss, B., Ginige, I., Shannon, L., Giribaldi, B., Murphy-Beiner, A., Murphy, R., Baker-Jones, M., Martell, J., Nutt, D.J., Carhart-Harris, R.L., and Erritzoe, D. (2024). Personality change in a trial of psilocybin therapy v. escitalopram treatment for depression. Psychol. Med. *54*, 178–192. https://doi.org/10.1017/S0033291723001514.
30. Daws, R.E., Timmermann, C., Giribaldi, B., Sexton, J.D., Wall, M.B., Erritzoe, D., Roseman, L., Nutt, D., and Carhart-Harris, R. (2022). Increased global integration in the brain after psilocybin therapy for depression. Nat. Med. *28*, 844–851. https://doi.org/10.1038/s41591-022-01744-z.

31. Stoliker, D., Egan, G.F., Friston, K.J., and Razi, A. (2022). Neural Mechanisms and Psychology of Psychedelic Ego Dissolution. Pharmacol. Rev. *74*, 876–917. https://doi.org/10.1124/pharmrev.121.000508.
32. Simonsson, O., Hendricks, P.S., Chambers, R., Osika, W., and Goldberg, S.B. (2023). Prevalence and associations of challenging, difficult or distressing experiences using classic psychedelics. J. Affect. Disord. *326*, 105–110. https://doi.org/10.1016/j.jad.2023.01.073.
33. Davis, M. (1992). The Role Of The Amygdala In Fear And Anxiety. Annu. Rev. Neurosci. *15*, 353–375. https://doi.org/10.1146/annurev.neuro.15.1.353.
34. Abuhasan, Q., Reddy, V., and Siddiqui, W. (2021). Neuroanatomy, Amygdala (StatPearls).
35. Ahrens, S., Wu, M.V., Furlan, A., Hwang, G.R., Paik, R., Li, H., Penzo, M.A., Tollkuhn, J., and Li, B. (2018). A central extended amygdala circuit that modulates anxiety. J. Neurosci. *38*, 5567–5583. https://doi.org/10.1523/JNEUROSCI.0705-18.2018.
36. Ruiz, N.A.L., Del Ángel, D.S., Olguín, H.J., and Silva, M.L. (2018). Neuroprogression: The hidden mechanism of depression. Neuropsychiatr. Dis. Treat. *14*, 2837–2845. https://doi.org/10.2147/NDT.S177973.
37. Roseman, L., Demetriou, L., Wall, M.B., Nutt, D.J., and Carhart-Harris, R.L. (2018). Increased amygdala responses to emotional faces after psilocybin for treatment-resistant depression. Neuropharmacology *142*, 263–269. https://doi.org/10.1016/j.neuropharm.2017.12.041.
38. Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L.A., and Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology (Berl) *204*, 361–373. https://doi.org/10.1007/s00213-009-1466-y.
39. Bauer, H.F., Delling, J.P., Bockmann, J., Boeckers, T.M., and Schön, M. (2022). Development of sex- and genotype-specific behavioral phenotypes in a Shank3 mouse model for neurodevelopmental disorders. Front. Behav. Neurosci. *16*, 1051175. https://doi.org/10.3389/fnbeh.2022.1051175.
40. Jones, N.T., Zahid, Z., Grady, S.M., Sultan, Z.W., Zheng, Z., Banks, M.I., and Wenthur, C.J. (2020). Delayed Anxiolytic-Like Effects of Psilocybin in Male Mice Are Supported by Acute Glucocorticoid Release. Preprint at bioRxiv *2*. https://doi.org/10.1101/2020.08.12.248229.
41. Hesselgrave, N., Troppoli, T.A., Wulff, A.B., Cole, A.B., and Thompson, S.M. (2021). Harnessing psilocybin: Antidepressant-like behavioral and synaptic actions of psilocybin are independent of 5-HT2R activation in mice. Proc. Natl. Acad. Sci. USA *118*, e2022489118. https://doi.org/10.1073/pnas.2022489118.
42. Moliner, R., Girych, M., Brunello, C.A., Kovaleva, V., Biojone, C., Enkavi, G., Antenucci, L., Kot, E.F., Goncharuk, S.A., Kaurinkoski, K., et al. (2023). Psychedelics promote plasticity by directly binding to BDNF receptor TrkB. Nat. Neurosci. *26*, 1032–1041. https://doi.org/10.1038/s41593-023-01316-5.
43. Kedra, M., Banasiak, K., Kisielewska, K., Wolinska-Niziol, L., Jaworski, J., and Zmorzynska, J. (2020). TrkB hyperactivity contributes to brain dysconnectivity, epileptogenesis, and anxiety in zebrafish model of Tuberous Sclerosis Complex. Proc. Natl. Acad. Sci. USA *117*, 2170–2179. https://doi.org/10.1073/pnas.1910834117.
44. Bullitt, E. (1990). Expression of C-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J. Comp. Neurol. *296*, 517–530. https://doi.org/10.1002/cne.902960402.
45. Kataoka, M., Yamamori, S., Suzuki, E., Watanabe, S., Sato, T., Miyaoka, H., Azuma, S., Ikegami, S., Kuwahara, R., Suzuki-Migishima, R., et al. (2011). A single amino acid mutation in SNAP-25 induces anxiety-related behavior in mouse. PLoS One *6*, e25158. https://doi.org/10.1371/journal.pone.0025158.
46. Letellier, M., Levet, F., Thoumine, O., and Goda, Y. (2019). Differential role of pre-and postsynaptic neurons in the activity-dependent control of synaptic strengths across dendrites. PLoS Biol. *17*, e2006223. https://doi.org/10.1371/journal.pbio.2006223.
47. Roseman, L., Nutt, D.J., and Carhart-Harris, R.L. (2017). Quality of acute psychedelic experience predicts therapeutic efficacy of psilocybin for treatment-resistant depression. Front. Pharmacol. *8*, 974. https://doi.org/10.3389/fphar.2017.00974.
48. Jones, N.T., Zahid, Z., Grady, S.M., Sultan, Z.W., Zheng, Z., Razidlo, J., Banks, M.I., and Wenthur, C.J. (2023). Transient Elevation of Plasma Glucocorticoids Supports Psilocybin-Induced Anxiolysis in Mice. ACS Pharmacol. Transl. Sci. *6*, 1221–1231. https://doi.org/10.1021/acsptsci.3c00123.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti c-fos	Santa Cruz Biotechnology	Cat#:sc-52; RRID: AB_2106783
Alexa 488-labeled anti-rabbit	Jackson laboratories	Cat#:111-545-144; RRID: AB_2338052
Chemicals, peptides, and recombinant proteins		
psilocybin	Lipomed	N/A
ketanserin	Sigma-Aldrich	Cat#:S006
hoescht	Sigma-Aldrich	Cat#:33258
Experimental models: Organisms/strains		
C57BL/6J mice	Invigo labs	N/A
Software and algorithms		
Ethovision-XT	Noldus	https://www.noldus.com/ethovision-xt
String	String Consortium	https://string-db.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Evan Elliott (evan.elliott@biu.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data used to perform analyses have been included as publicly available supplemental information.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

8-10 weeks old C57BL/6J male mice (supplied by Invigo Laboratories, Rehovot, Israel) were housed according to Federation of Laboratory Animal Science Associations (FELASA) guidelines. Mice were maintained in a vivarium at 22 C with a reversed light-cycle (lights on at 19:00 h, off at 07:00 h). Food and water were provided *ad libitum*, except during behavioral testing. Testing occurred between 9:00 and 16:00 h. Littermate mice were randomly selected to experimental groups. All experimentation was approved by the Institutional Animal Care and Use Committee under protocol number 42-06-2021.

METHOD DETAILS

Pharmacological treatment of mice

Compounds used were psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine) (psi-411-fb-10, Lipomed) and ketanserin 3-(2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl)-2,4(1H,3H)-quinazolinedione (+)-tartrate salt (S006-10MG, Sigma Aldrich). All compounds were dissolved in Phosphate-buffered saline (PBS) (02-023-5A, Sartorius) and administered i.p. in a volume of 200 μL. For studies of dose-dependent effect of psilobyin, separate mice were injected i.p. with 1 mg/kg, 3 mg/kg, or 5 mg/kg psilocybin, or PBS vehicle. Mice were subjected to marble burying, open field, dark light, or elevated plus maze 30 min to 1 h after psilocybin treatment. In ketanserin experiments, Mice were i.p. injected with 3 mg/kg ketanserin or PBS 30 min prior to i.p. injection with psilocybin or PBS.

Behavioral experiments

Mice were randomly distributed into their experimental groups. Analysis was blind to treatment, as it was done by automatic software. The experiments were recorded with the Panasonic WV-CL930 camera, and with the aid of the Ganz IR 50/50 Infrared panel, to enhance the detection of the mice. Mouse positioning and movement were analyzed by the Ethovision XT 10 (Noldus, Wageningen, The Netherlands) software.

Marble burying test

Mice were i.p. injected with the different treatments and 30 min after administration mice were placed in a compartment (20 × 40 cm). Twenty green glass marbles (15 mm in diameter) were arranged in a 4 × 5 grid on top of 5 cm clean bedding. Each mouse was placed in the compartment and was given 30 min exploration period, after which the number of marbles buried, was counted. "Buried" was defined as 2/3 covered by bedding. Testing was performed under dim light (20 lux).

Open field

Mice were i.p. injected with the different treatments and immediately after administration mice were placed in a corner of a square arena made from a Non-Glare Perspex (50 × 50 cm) which was illuminated at 120 lux for 30 min. During the trial we measured total distance moved to evaluate locomotion and time in center (25 × 25 cm) to evaluate anxiety-like behavior.

Dark light test

Mice were i.p. injected with the different treatments and 15 min after administration mice were placed in a small dark compartment and were allowed to cross through a small hatch into a larger compartment illuminated with 1200 lux. During the 5-min trial, time spent in the lighted zone and entry zone were measured to evaluate anxiety-like behavior and risk-assessing behavior.

Elevated plus maze

Mice were i.p. injected with the different treatments and 30 min after administration mice were placed in the center of the elevated plus maze and were allowed to roam between the closed and open arms which were illuminated in 20 lux. During the 5 min trial, time spent in open arms, closed arms and center was measured to evaluate anxiety-like behavior and risk-assessing behavior.

Head twitch response

Immediately after psilocybin administration mice were placed in a small compartment) 15 × 7.5 cm) which was illuminated at 120 lux for 15 min. The experiments were recorded with the iPad.9 camera and the videos were manually analyzed to measure the number of head twitch response performed by each mouse during the 15 min trail.

Immunostaining

Mice were i.p. injected with psilocybin in different concentrations and 60 min after administration mice were perfused with 4% paraformaldehyde (Sigma Aldrich) and then brains were dissected and incubated in 30% sucrose (s011-1kg, Tivan Biotech) for two days. 30μm slices sections were taken by sliding microtome (Epredia HM 430 Sliding Microtome). Slices were blocked for 1 h in blocking solution [10% horse serum (s-2000, Vector Laboratories), 0.3% Triton (T8787, Sigma Aldrich) and 1XPBS] and then incubated with c-Fos rabbit polyclonal IgG primary antibodies (1:300) (sc-52, Santa Cruz Biotechnology) for 48 h at 4°C. Slices were washed with incubated for 1 h with Alexa 488-labeled anti-rabbit secondary antibody (1:200) (Jackson immune research laboratories 111-545-144), stained for 5 min with Hoechst (1:1000) (33258 solution, Sigma Aldrich), and washed three times, followed by mounting. Five brains were perfused for each group, and 5-7 slices were taken from different brain region, dentate gyrus, PVN and basolateral amygdala images were taken on ZEISS Axio Scan.Z1 slide scanner and the number of c-Fos expressing cells was measured using ZEN blue image analysis software.

Phosphoprotein analysis

Mice were injected i.p. with either 3 mg/kg ketanserin, or PBS, and after 30 min were injected i.p. with either 5 mg/kg psilocybin or PBS. 15 min following psilocybin administration, mice were sacrificed by decapitation, brains were removed, and amygdala were dissected on dry ice. Frozen amygdala were sent to the Technion Proteomic Center.

Proteolysis

The proteins were extracted from the amygdala in 9M Urea, 400mM Ammonium bicarbonate and 10mM DTT following grinding (Omni TH Tissue Homogenizer) and two cycles of sonication. 20ug protein from each sample were reduced with 3mM DTT (60°C for 30 min), modified with 9mM iodoacetamide in 400mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 1M Urea, 50mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37°C. An additional second trypsinization was done for 4 h. The tryptic peptides were desalted using C18 tips (Top tip, Glygen) dried and re-suspended in 0.1% Formic acid.

For phosphor-enrichment 750ug were trypsinyzed similarly, cleaned on C18 oasis columns (Waters) and re-suspended in 40% Acetonitrile (ACN), 6% TFA (Trifluoroacetic acid), and enriched for phosphopeptides on titanium dioxide (TiO2) beads. Titanium beads were pre-washed

(80%ACN, 6% TFA) mix with the peptides for 10 min at 37°C, washed with 30%ACN with 3%TFA and 80%ACN, with 0.1%TFA. Bound peptides were eluted with 20%ACN with 325mM Ammonium Hydroxide followed by 80%ACN with 325mM Ammonium Hydroxide. The resulted peptides were desalted using C18 tips and analyzed by LC-MS-MS.

Mass spectrometry analysis

The peptides were resolved by reverse-phase chromatography on 0.075 × 180-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with different concentration of Acetonitrile with 0.1% of formic acid: a linear 180 min gradient of 5–28% acetonitrile followed by a 15 min gradient of 28–95% and 25 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 μL/min.

Mass spectrometry was performed by Q Executive HFX or for the phosphopeptides by Q Executive HF mass spectrometer (Thermo) in a positive mode (m/z 300–1800, resolution 120,000 for MS1 and 15,000 for MS2) using repetitively full MS scan followed by collision induced dissociation (HCD, at 27 normalized collision energy) of the 30 most dominant ions (>1 charges) selected from the first MS scan. A dynamic exclusion list was enabled with exclusion duration of 20 s.

The mass spectrometry data were analyzed using the MaxQuant softwaret 2.1 for peak picking and identification using the Andromeda search engine, searching against the mouse proteome from the Uniprot database with mass tolerance of 6 ppm for the precursor masses and the fragment ions. Methionine oxidation, phosphorylation (STY) and protein N-terminus acetylation were accepted as variable modifications and carbamidomethyl on cysteine was accepted as static modifications. Minimal peptide length was set to six amino acids and a maximum of two miscleavages was allowed. Peptide- and protein-level false discovery rates were filtered to 1% using the target-decoy strategy. The protein table was filtered to eliminate the identifications from the reverse database, and common contaminants. The data were quantified by label free analysis using the same software.

ANOVA was used to identify phosphoproteins that were significantly different among experimental groups. First, phosphopeptides were filtered for those that showed an ANOVA q value of less than 0.05. Second, phosphopeptides that had a significant difference (FDR<0.05) in the PBS-PBS group compared to the PBS-Psi group were identified. These are the phosphopeptides effected by psilocybin (psilocybin-effected). Among the psilocybin-effected phosphopeptides, those that showed a significant difference (FDR<0.05) between the PBS-Psi and ketanserin-Psi groups were defined as ketanserin-sensitive. Among the psilocybin-effected phosphopeptides, those that showed FDR>0.3 between PBS-Psi and ketanserin-Psi groups were defined at ketanserin-insensitive. All data are available as Table S1.

Protein-protein Interaction network analysis

Gene lists of phosphorylated proteins (ketanserin-sensitive and ketanserin-insensitive) from Phosphoproteomics-FDR analysis were loaded onto the STRING online database (version 12), and high confidence protein interactions (0.7) were determined. Then, the yielded networks were further analyzed to produce k-means clustering: the network topography (number of interactions, strength, and significant) was based on the confidence score calculated by STRING.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis and quantification for Mass spectrometry analysis was done using Perseus 1.6.2.2 software. All other statistical analysis was performed by SPSS.23 Software and GraphPad-Prism.9 Software. The outcome of all ANOVA analyses performed in this manuscript is found in Table S2.