Received: 1 June 2022 | Revised: 16 August 2022 | Accepted: 4 September 2022

DOI: 10.1002/syn.22253

SYNAPSE WILEY

RESEARCH ARTICLE

Individual differences in the positive outcome from adolescent ketamine treatment in a female mouse model of anorexia nervosa involve drebrin A at excitatory synapses of the medial prefrontal cortex

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Funding information
National Science Foundation, Grant/Award Numbers: REU1460880, REU1950649; New York University Dean's Undergraduate Research Fund; National Institutes of Health, Grant/Award Numbers: R21MH105846, R01NS066019-01A1, R01NS047557-07A1, EY13079; Klarman Family Foundation Grant Program in Eating Disorders Research; Fulbright Scholarship; Vulnerable Brain Project; New York University Research Challenge Fund

Abstract

Anorexia nervosa (AN) is a mental illness with the highest rates of mortality and relapse, and no approved pharmacological treatment. Using an animal model of AN, called activity-based anorexia (ABA), we showed earlier that a single intraperitoneal injection of ketamine at a dose of 30 mg/kg (30mgKET), but not 3 mg/kg (3mgKET), has a long-lasting effect upon adolescent females of ameliorating anorexia-like symptoms through the following changes: enhanced food consumption and body weight; reduced running and anxiety-like behavior. However, there were also individual differences in the drug's efficacy. We hypothesized that individual differences in ketamine's ameliorative effects involve drebrin A, an F-actin-binding protein known to be required for the activity-dependent trafficking of NMDA receptors (NMDARs). We tested this hypothesis by electron microscopic quantifications of drebrin A immunoreactivity at excitatory synapses of pyramidal neurons (PN) and GABAergic interneurons (GABA-IN) in deep layer 1 of prefrontal cortex (PFC) of these mice. Results reveal that (1) the areal density of excitatory synapses on GABA-IN is greater for the 30mgKET group than the 3mgKET group; (2) the proportion of drebrin A+ excitatory synapses is greater for both PN and GABA-IN of 30mgKET than 3mgKET group. Correlation analyses with behavioral measurements revealed that (3) 30mgKET's protection is associated with reduced levels of drebrin A in the cytoplasm of GABA-IN and higher levels at extrasynaptic membranous sites of PN and GABA-IN; (5) altogether pointing to 30mgKET-induced homeostatic plasticity that engages drebrin A at excitatory synapses of both PN and GABA-IN.

KEYWORDS
anorexia nervosa, drebrin, ketamine, prefrontal cortex

1 | INTRODUCTION

Anorexia nervosa (AN) is a deadly eating disorder characterized by severe, self-imposed food restriction, severe anxiety, manifested through a fear of gaining weight, and body dysmorphia, according to The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (APA, 2013; Kaye et al., 2004). Excessive exercise is another core symptom of AN, even though it is not listed by the DSM-5 (Davis, 1999; le Grange & Eisler, 1993;

Peñas-Lledó et al., 2002). The combination of these characteristics leads to severe weight loss (Arcelus et al., 2011; Birmingham et al., 2005) and the highest mortality rate of any psychiatric illness (Arcelus et al., 2011). AN is commonly comorbid with depression (Mandal et al., 2019), including suicidal ideation, and anxiety disorders, including obsessive-compulsive disorder and post-traumatic stress disorder (Bühren et al., 2014; Bulik et al., 2008; Hughes, 2012; Kaye et al., 2004; Swinbourne et al., 2012; Woodside & Staab, 2006). There are currently no FDA-approved pharmacotherapy treatments for AN, and little is known about its etiology (Hermens et al., 2020).

Ketamine is prescribed off-label for anxiety and AN, as well as other mood disorders (Banov et al., 2020; Carboni et al., 2021; Diazgranados et al., 2010; Mandal et al., 2019; Walsh et al., 2022). Clinical studies have shown subanesthetic doses of ketamine to be a fast and sustained antidepressant both in humans (Berman et al., 2000; Price et al., 2009; Zarate et al., 2006) and animals, unlike traditional antidepressants (Autry et al., 2011; Browne & Lucki, 2013; N. Li et al., 2010; Maeng et al., 2008; Zanos et al., 2016). A research group led by Mills (Mills, 1998) had a particular focus on AN as a compulsive behavior disease, characterizing it as a habitual recall of anorexic thoughts. They hypothesized that ketamine could disrupt compulsive thoughts. Indeed, their clinical study of chronic and treatment resistant AN subjects showed 9 out of 15 patients experiencing prolonged remission with drastic decreases in compulsivity depression scores (Mills, 1998). A case study regarding a patient experiencing severe and chronic (15 years) AN resulted in complete and sustained remission after receiving intravenous ketamine infusions in combination with a ketogenic diet (Scolnick et al., 2020). This promising case study has been followed by another, involving five chronic AN subjects (Calabrese, 2022). Another case study highlights an extreme and chronic case of a bulimia nervosa patient reaching complete and enduring remission after ketamine-assisted psychotherapy (Ragnhildstveit et al., 2021). Although bulimia is different in its disordered eating classification, it shares the same psychopathology of heightened anxiety and maladaptive behaviors focusing on weight-maintenance (Murphy et al., 2010). Studies such as these provide hope for patients with this tragic disease, and their families. They provide reasons for further studies to understand the mechanisms behind these remarkable recoveries involving ketamine.

Activity-based anorexia (ABA) is a commonly used animal model to study the neurobiological basis of AN, allowing new understandings of the disease's etiology and potential treatments (Aoki, 2021; Aoki & Sherpa, 2017; Chowdhury et al., 2015; Gutierrez, 2013). ABA captures four key characteristics of AN (Gutierrez, 2013): (1) exaggerated voluntary exercise (Wable et al., 2015); (2) food restriction (FR), which is seemingly voluntary, as mice run on the wheel rather than eat during food availability hours (Wable et al., 2015); both of which yield (3) severe weight loss, sometimes lethal; and (4) heightened anxiety (Chen et al., 2018; Hughes 2012). ABA hyperactivity is inducible in any wild-type adolescent rodent, if food availability is limited to 1–2 h per day (Chowdhury et al., 2015; Chowdhury et al., 2013). There are individual differences in the gain of resilience when mice undergo ABA induction, similar to humans in the way only some are vulnerable to AN. These individual differences in vulnerability are seen even within a single litter of mice (Chowdhury et al., 2013), indicating that susceptibility to this disease is very individualized.

Using ABA as the animal model of AN, it was observed that a single systemic injection of ketamine (30mgKET) in mid-adolescence reduced ABA vulnerability across all four measures: increased food intake and body weight, decreased wheel running, and decreased anxiety-like behavior (Chen et al., 2018). In contrast, the lower dose of ketamine (3mgKET) was less efficacious in reducing ABA vulnerability. Moreover, there were individual differences in drug efficacy even among the 30mgKET group (Chen et al., 2018). We asked whether the dose-dependent group difference as well as the individual differences in behavioral responses to the efficacious dose correspond to individual differences in synaptic protein expression patterns. To answer this question, brain tissues of ABA animals that underwent the above ketamine treatments during mid-adolescence were collected and preserved for correlative analyses of behavior and molecular characteristics of excitatory synapses 20 days later, after having experienced a second ABA induction (ABA2) and recovery from ABA2.

As to where within the brain to seek correlates to behavior, we began by analyzing the prefrontal cortex (PFC). One reason is that the PFC is important in decision-making (Funahashi, 2017). In the case of ABA, the decision is to run or to eat. PFC is also important in anxiety regulation, as it is the interface between limbic and cortical structures, with vast projections to the hippocampus and amygdala (Arco & Mora, 2009; Groenewegen & Uylings, 2000). PFC has extensive projections to the amygdala, making it an important gateway in the extinction of fear-based memories (Herry & Garcia, 2002; Milad et al., 2004) and extinction maintenance (Herry & Garcia, 2003; Phelps et al., 2004). Additionally, abnormal PFC activity is seen in patients with AN. In particular, an increase in PFC activity is seen upon presentation of food or body images, both of which are anxiety-provoking in AN (Brooks et al., 2012; Castellini et al., 2013; Miyake et al., 2010; Scharner & Stengel, 2019; Uher et al., 2004). Abnormal functional connectivity of PFC to dorsal striatum is also seen in patients with AN (Foerde et al., 2015). We have recently shown that chemogenetic activation of the PFC-to-striatum pathway exacerbates food restriction-evoked hyperactivity (Santiago et al., 2021), while GABAergic inhibition within PFC of pyramidal neurons (PN) projecting to dorsal raphe underlies reduced food consumption of animals undergoing ABA (Du et al., 2022).

Within PFC, layer 1 is implicated in stress-related mood disorders, with chronic stress significantly reducing activity-dependent synaptic plasticity (Negrón-Oyarzo et al., 2015). Layer 1 is a cortical layer with the highest synapse density (DeFelipe , 1999) and is a site of integration of excitatory inputs from PN of all layers as well as from cortico-cortical, thalamo-cortical and amygdalo-cortical afferents (Anastasiades et al., 2020; Little & Carter, 2013; Muralidhar et al., 2014). Ketamine may facilitate mediodorsal thalamic nucleus's synaptic drive onto PN belonging to medial prefrontal cortex (mPFC) via blockade of GluN2B-containing NMDA receptor (NMDAR) on PN (Miller et al., 2017). Mediodorsal thalamic nuclei primarily drive interneurons in layer 1b, specifically of the vasoactive intestinal peptide-expressing (VIP+ IN) subtype of GABAergic interneurons (GABA-IN) (VIP+ GABA-IN) (Anastasiades et al., 2020). These VIP+ GABA-IN then engage in a local disinhibitory circuit, which yields excitation of the mPFC circuit,

thereby potentially able to support synaptic plasticity of PN across the layers in PFC (Anastasiades et al., 2020). We therefore focused our synapse analysis on layer 1b.

Ketamine is known to be a noncompetitive antagonist of NMDARs of the glutamate system, making the glutamatergic neurotransmitter system the focus of this study. Within layer 1b of PFC, we analyzed two cell types engaged in excitatory synaptic transmission by electron microscopy (EM): PN that utilize glutamate as the neurotransmitter and GABA-IN. PN receive excitatory synaptic inputs at dendritic spines, while GABA-IN receive excitatory synaptic inputs at dendritic shafts (White & Keller, 1989), enabling us to distinguish the cell types on which excitatory synapses are found by EM.

Drebrin A is the adult isoform of an F-actin-binding protein enriched at excitatory synapses (Aoki et al., 2005; Hayashi et al., 1996) and involved in the NMDAR activity-dependent accumulation and removal of NMDARs at synapses, contributing to homeostatic synaptic plasticity (Aoki et al., 2009). The mechanism of action of ketamine is hypothesized to also involve homeostatic synaptic plasticity that is evoked following antagonism of NMDARs (Kavalali & Monteggia, 2020). This commonality prompted us to examine the possible role of drebrin A in ketamine-evoked gain of resilience to ABA. The rise of drebrin A coincides with the developmental stage of robust synaptogenesis and the conversion of nascent filopodia to stable dendritic spines, while the decline of drebrin A precedes cognitive impairment associated with Alzheimer's disease (Aoki et al., 2017). These are additional key findings suggesting that drebrin A is an important participant in synaptic plasticity. Membranous drebrin A are likely to reflect their role of drebrin A in stabilizing NMDAR to synaptically and extrasynaptically functional sites, while cytoplasmic drebrin A may reflect the sequestration of NMDAR away from active sites (Aoki et al., 2017). EM enabled us to discriminate between these subcellular locations of drebrin A at excitatory synapses. We hypothesized that individual differences in ketamine-responsiveness during and following ABA induction is due to individual differences in brain changes evoked by ketamine revolving changes in the distribution pattern of drebrin A relative to excitatory synapses in layer 1b of PFC. We present EM data supporting this hypothesis.

2 | MATERIALS AND METHODS

2.1 | Animals

Sixteen female C57Bl/6 J wild-type littermates were bred at the university's animal facility. All procedures involving the use of animals were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and also approved by the Institutional Animal Care and Use Committee of the authors' University (Animal Welfare Assurance No. A3317-01). All animals were housed in a room kept on a 12:12 light–dark cycle (lights on at 0700 h). Food and water were available ad libitum, until experimental procedures began. After weaning at postnatal day 25 (P25), animals of the same sex were group-housed 2–4 per cage.

2.2 | Activity-based anorexia

The ABA protocol begins by acclimating single-housed animals to a wheel, providing ad libitum opportunity for voluntary exercise. Food and water are also available ad libitum. Voluntary wheel running distance and duration are measured 24/7, while food consumption is recorded once or more daily.

ABA induction then begins by limiting the hours of food access to 2 h per day, without restricting the amount of food available during these hours. Wheel access and water access remain ad libitum. This starvation period is sufficient to induce significant body weight loss, but not severe enough to cause death. FR lasts 3 days, followed by a 7-day recovery period. A second round of ABA (ABA2) follows, modeling a relapse period. Interestingly, it is observed that after significant body weight loss, these adolescent mice increase wheel activity significantly. Mice run rather than eat during their 2 h of food availability, allowing us to refer to this phenotype as voluntary food restriction. This protocol is further described elsewhere (Aoki 2021; Chowdhury et al., 2015) and is summarized in Figure 1.

On P42 corresponding to the second day of the first ABA, as summarized in Figure 1, animals were pseudo-randomly assigned to three treatment groups. Two of the groups were injected with ketamine at a dose of 3 mg/kg (3mgKET) or 30 mg/kg (30mgKET). A third group was injected with vehicle (saline). The three groups were counterbalanced to ensure that the groups' average body weight and baseline running were not significantly different. Ketamine (Henry Schein, Melville, NY), diluted with saline, was injected intraperitoneally (i.p.) 1 h prior to the feeding time on P42. Vehicle control animals received i.p. saline at the same time. Body weight, wheel activity, and food consumption were recorded daily throughout the process, as well as before and after food-availability periods. These behavioral experimental data have been published previously (Chen et al., 2018) and were shared with the current authors as raw and processed data.

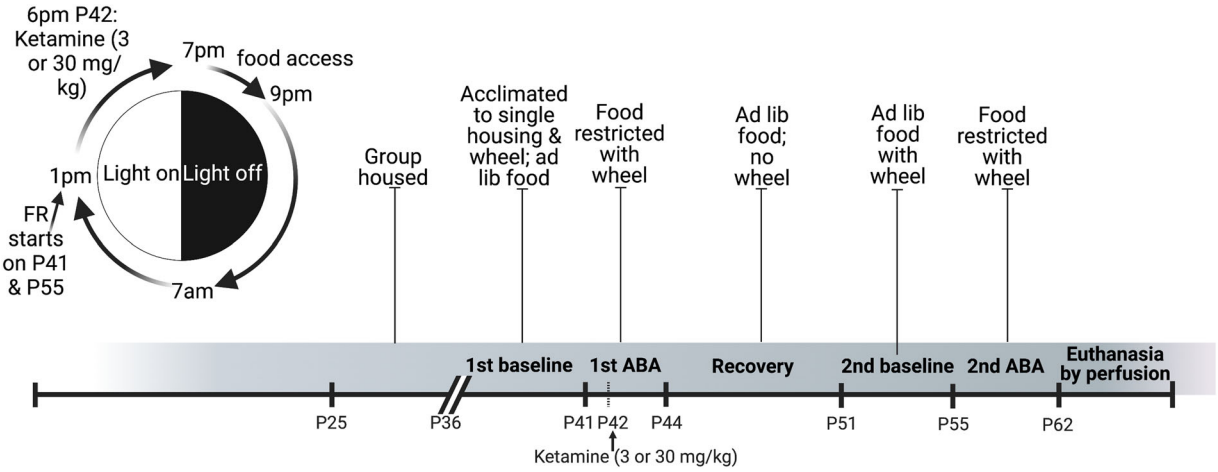

FIGURE 1 Activity-based anorexia (ABA) paradigm and ketamine injection timeline and scheme of ABA design and timing of drug injections. All female C57Bl/6 J mice experience repeated ABA exposures, the first ABA in mid-adolescence (P36–P44) and the second ABA in late-adolescence (P51–P59). On P42, the second day of the first ABA, female mice were assigned to different ketamine-dose groups that received ketamine (3 or 30mgKET) injection at 6 p.m. 1 h before the 2 h food availability (7–9 p.m.). Adapted from "Mouse Experimental Timeline", by BioRender.com (2022)

2.3 | Tissue preparation for EM immunocytochemistry

Female mice (N = 8 for 30mgKET and N = 8 for 3mgKET) were euthanized on the morning of P63 ±1 day. The mice were deeply anesthetized with urethane (i.p. 1000–1500 mg/kg body weight), then vaginally smeared to assess the estrous phase. Animals were then transcardially perfused with 50 ml of phosphate-buffered saline (PBS, 0.01 M phosphate buffer plus 0.9% sodium chloride) containing Heparin (20 U/ml), followed without pause with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde (PFA, EM Sciences, Hatfield, PA, USA). The brain of each mouse was removed from the skull and stored in 4% paraformaldehyde in 0.1 M phosphate buffer at room temperature for 3 days and then in a cold room set to 3°C for 2 years. The brains were blocked into coronal plane slabs, 3 mm thick. Each block was then further sliced in the coronal plane using a vibrating microtome at a thickness set to 50 μm (Leica VT1000M; Leica Microsystems GmbH, Wetzlar, Germany). Coronal sections containing optimal cross sections of the mPFC (AP Bregma 1.7–2.34, centered at 1.94) were selected.

2.4 | Drebrin A immunoreactivity/silver-intensified gold tissue processing: Materials and the procedure

The primary polyclonal antibody was directed against drebrin A, produced in rabbit against the amino acid sequence unique to the adult isoform of drebrin A (residues 327–338), which is identical in mouse, rat, and human. The antibody was a generous gift of Dr. Tomoaki Shirao from Gunma University School of Medicine, originally obtained from Immuno-Biological Laboratories (Cat No. 28023, lot 1F-213, Antibody Registry #AB_10706147). Specificity of this antibody has been demonstrated, based on a single band recognized by western blot of mouse whole brain homogenates and synaptosomes (Aoki et al., 2005) and elimination of immunoreactivity when applied to brains of mice with global knockout of drebrin A (Aoki et al., 2009). The secondary antibody was obtained from Electron Microscopic Sciences (EMSciences) (goat anti-rabbit IgG conjugated with ultra-small (0.8 nm) gold particles [Cat No. 25101, lot 800.011]). The silver intensification kit, used to enlarge the 0.8 nm colloidal gold particles, was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA).

After testing immunoreactivity of brain tissue to the anti-drebrin A antibody at dilutions ranging from 1:10 to 1:1000, it was determined that 1:400 was optimal in providing maximal immuno-labeling and free of background. As for the discrimination of specific versus background labeling, a previously published study established that drebrin A immunoreactivity occurs almost entirely on the postsynaptic side of excitatory synapses and minimally at inhibitory synapses or in astrocytes (Aoki et al., 2005; Aoki et al., 2017). We further verified the absence of immunolabeling of brain section obtained from mice with genetic deletion of drebrin A, as was tested previously (Aoki et al., 2009) (data not shown). Thus, immunolabeling free of background was assessed based on electron microscopic verification of this immunolabeling pattern.

Prior to the labeling procedure, sections were incubated in 1% hydrogen peroxide in PBS for 30 min and rinsed in PBS. They were then incubated in a blocking buffer, 1% bovine serum albumin in PBS-azide, to minimize nonspecific antibody labeling. The immunocytochemical procedure commenced by incubating the free-floating sections in PBS azide containing 1% bovine serum albumin (Sigma Chem) and 1:400 dilution of the rabbit primary antibody directed against the drebrin A protein. The free-floating sections were agitated continuously at room temperature for 67 h in

the presence of the anti-drebrin A antibody, after which time, excess unbound primary antibodies were removed by rinsing in PBS. Sections were then incubated overnight at room temperature in the goat secondary antibody, consisting of an anti-rabbit IgG conjugated to 0.8 nm colloidal gold particles. On the following day, excess unbound secondary antibodies were removed by rinsing in PBS, then post-fixed by immersing the sections in PBS containing 2% glutaraldehyde (EMSciences EM grade) for 10 min. After rinsing in PBS, sections were stored overnight at 4°C, then processed for silver-intensification of the colloidal gold particles, to enlarge the gold particles to sizes detectable by EM.

The silver-intensified colloidal gold particles (SIG) ranged in sizes from 10 to 300 nm, even though all procedures were run strictly in parallel. The silver-intensified sections were processed osmium-free (Phend et al., 1995), to avoid oxidation of the SIG particles. The heavy metals used to generate contrast were uranyl acetate, which also served to improve ultrastructural preservation (Lózsa, 1974; Terzakis, 1968), iridium tetrabromide, and Reynold's lead citrate. Vibratome sections were then infiltrated with EMBED-812, flat-embedded between two sheets of Aclar plastic, then ultrathin-sectioned at a thickness setting of 70 nm at a cutting plane tangential to the vibratome-cut surface, to maximize capture of the synaptic neuropil from section surfaces, where penetration by immunoreagents was maximal.

2.5 | EM image collection and analysis

2.5.1 | Image collection procedure

From the brain of each animal, at least 200 asymmetric (presumably excitatory (Peters et al., 1991)) synapses in layer 1b of prelimbic (PrL) mPFC were analyzed at a direct magnification of 30,000×. Analysis was restricted to layer 1b by ensuring that the sampled area was at least 25 mm away from cortical surface and by the absence of pyramidal neurons' cell bodies in the neuropil. Cell bodies belonging to pyramidal neurons exhibit mostly smooth nuclear envelopes, while cell bodies of GABA-INs (which can be found located in layer 1b) have multiple deeply invaginated nuclear envelopes (White & Keller, 1989). This distinctive feature of the nuclear envelopes enabled detection of the transition between layer 1b and layer 2, the latter of which could contain pyramidal neurons. Cortical surface was readily identified by the thin lining of glia limitans at the edge of tissue (Peters et al., 1991). Digitized images were captured using AMT's XR80 CCD camera system (Boston, MA, USA) connected to the JEOL 1200XL EM. Excitatory axo-spinous synapses belonging to PNs were identified using criteria described before (Aoki et al., 2005). Specifically, this consisted of a prominence of thick postsynaptic density (PSD), lack of mitochondria or microtubules, and parallel alignment of the plasma membrane associated with the PSD with that of the axon terminal containing clusters of vesicles. Dendritic shafts receiving asymmetric synapses, presumably of GABA-IN (White & Keller, 1989), were sampled from PrL of PFC of each animal as well. Postsynaptic dendritic shafts were identified by the presence of mitochondria and microtubules, prominence of a thick PSD, and parallel alignment of plasma membrane associated with the PSD with that of the axon terminal containing clusters of vesicles. All excitatory synapses on spines and shafts encountered at the surface-most regions of vibratome-cut surfaces of sections were analyzed for the subcellular locations and levels of immunoreactivity to drebrin A, (see Figure 2, detailed further below), strictly in the order of encounter, to ensure randomness of sampling. The electron microscopist remained blind to the ketamine dosing condition and behavior of the animal throughout the sessions for EM image capture and during quantitative analyses.

2.5.2 | Method for quantifying synapse density and drebrin A levels

The average areal axo-spinous synaptic density for each animal was calculated by quantifying the number of axo-spinous PSDs encountered, divided by the total tissue area analyzed for each animal. The same was done to calculate for axo-shaft average areal synaptic density of each animal. For both the dendritic spines and dendritic shafts associated with excitatory synapses, the location of SIG was noted as being at or near (<10 μm away from) PSD, at or near the plasma membrane extrasynaptically, or intracellular, also referred to as cytoplasmic (Figure 2). The number of synapses with each type of SIG locations as well as the total number of SIG particles per synapse were recorded.

The percentage of axo-spinous synapses labeled postsynaptically with membranous SIG for each animal was calculated by quantifying the number of axo-spinous synapses labeled with membranous SIG particles and dividing by the total number of axo-spinous synapses. The same procedure was done for all locations of SIG particles: membranous, extrasynaptic, PSD, and intracellular for both axo-spinous and axo-shaft synapses. The level of drebrin A at axo-spinous synapses labeled postsynaptically with membranous SIG for each animal was calculated by quantifying the number of membranous SIG particles across all axo-spinous synapses encountered and dividing by the total number of axo-spinous synapses. The same procedure was done for all locations of SIG particles: membranous, extrasynaptic, PSD, and intracellular for both axo-spinous and axo-shaft synapses.

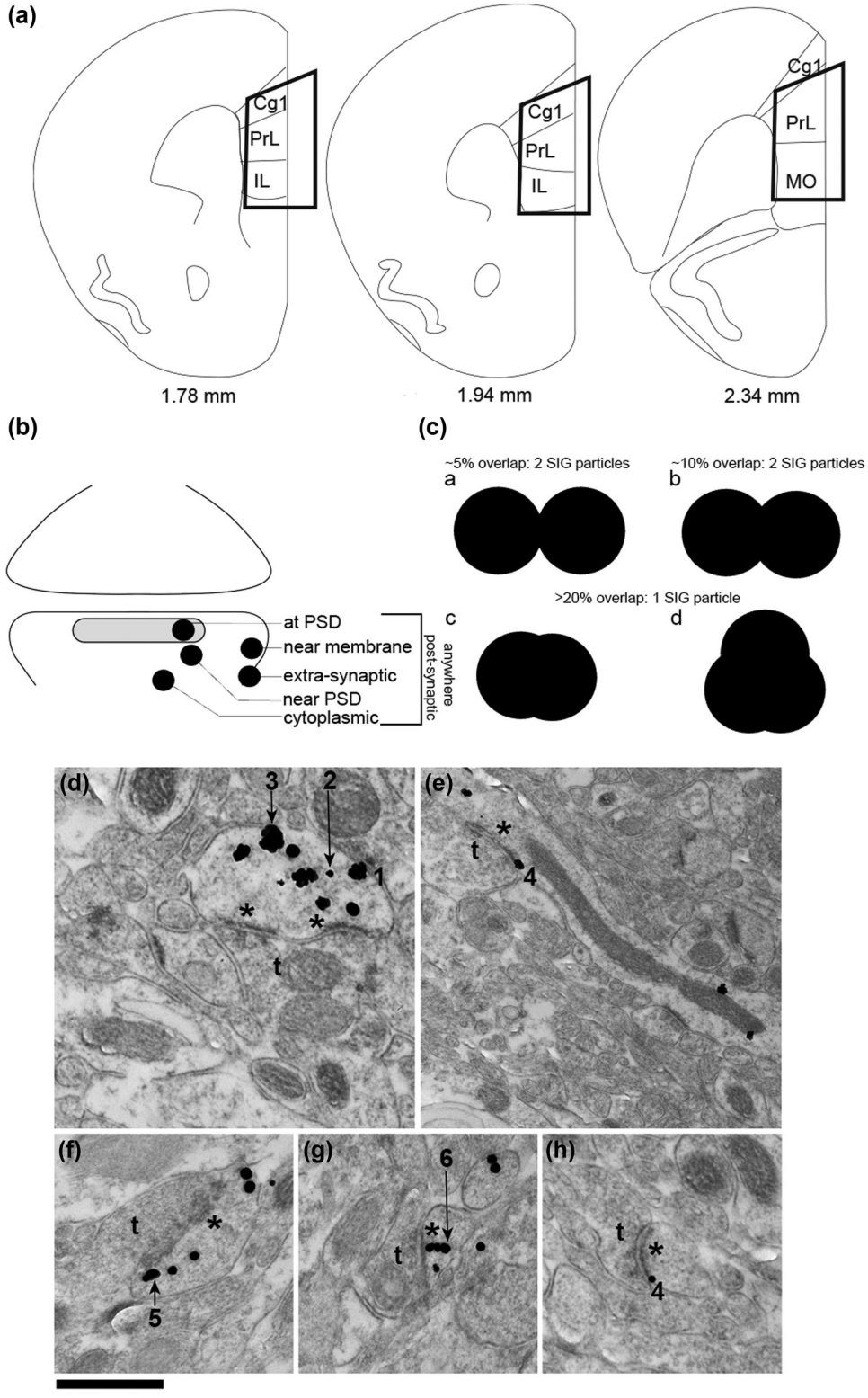

FIGURE 2 Categorization of subcellular sites in the vicinity of axo-spinous and axo-shaft asymmetric synapses where drebrin A immunoreactivity was observed by electron microscopy (EM). (a) Coronal sections containing optimal cross sections of the medial prefrontal cortex: AP Bregma 1.4–2.34 mm, centered at 1.94. Trapezoid outlines region of brain which was ultrathin-sectioned. The image (b) describes the categorization of subcellular location of silver-intensified colloidal gold (SIG) particles. The image (c) shows examples of how the SIG particles were counted. Example (a) would be classified as two separate particles; example (b) has slight enough overlap and would be classified as two separate particles (~10% overlap); examples (c) and (d) were classified as 1 particle, as the overlap is too much to decipher between a smudged particle and separate particles. In (D–H), examples of micrographs taken from tissue immunolabeled for drebrin A are shown. The presynaptic sides are

(Continues)

FIGURE 2 (Continued)
indicated by *t* for the axon terminal. Calibration bar is equal to 600 nm and applies to panels C, E, F, and G. Calibration bar is equal to 1,034 nm applying only to panel D. The numbers depict SIG associated with asymmetric synapses. All micrographs are depicting axo-spinous excitatory synapses, except for panel D which shows an excitatory synapse onto a dendritic shaft. *Represents the postsynaptic density. The subcellular position and categorization for each particle in these examples were categorized as follows: 1 at plasma membrane; 2 cytoplasmic, singular particle; 3 plasma membrane, too much overlap to decipher multiple particles, so counted as 1 cluster; 4 SIG particles at the postsynaptic density; 5 near the postsynaptic density; 6 represents three overlapping particles counted separately

2.6 | Statistical analyses

Normality of the distribution of measures was tested using the D'Agostino & Pearson omnibus normality test, Shapiro–Wilk normality test, and Kruskal–Wallis test. One-way analysis of variance was used to evaluate the significance of the differences among the two treatment groups, followed by Fisher's least significant difference (LSD) post hoc analysis. Unpaired *t*-test was used to evaluate the significance of the differences among the two treatment groups across days, followed by Fisher's LSD post hoc analysis. All the results are expressed as mean ± SEM, with *p*-values <.05 considered statistically significant. GraphPad Prism Version 9.20 was used.

2.7 | Preparation of figures

Digital images captured by EM were adjusted for gain, brightness, and scale and cropped or rotated using Adobe Photoshop version 23.3.0 of Creative Clouds. Graphs were plotted using GraphPad Prism Version 9.20.

3 | RESULTS

EM-immunocytochemistry was performed to discern drebrin A at and near the plasma membrane of excitatory synapses that may be involved in promoting the accumulation of glutamate receptors at functional sites, and, conversely, the cytoplasmic pool of drebrin A that may be involved in sequestering glutamate receptors away from functional sites. Moreover, the location of excitatory synapses was distinguished as those associated with dendritic spines, presumably of PN (White & Keller, 1989) versus those on dendritic shafts, presumably of GABA-IN (White & Keller, 1989).

3.1 | Dose of ketamine that enhanced resilience to ABA enhanced GABA-IN synaptic density

EM analysis was conducted for excitatory synapses, strictly in the order of encounter under magnification of 30,000×, following systematic sweeps of the synaptic neuropil at surface-most regions of vibratome sections, while also maintaining the region of interest.

Layer 1b of PFC of the 30mgKET group exhibited an increase in axo-shaft excitatory synaptic density presumably of GABA-IN (White & Keller, 1989) compared to the 3mgKET group (Figure 3a). The 30mgKET group exhibited no change in axo-spine excitatory synaptic density, presumably of PN (White & Keller, 1989), compared to the 3mgKET group (Figure 3b). We asked whether the apparent increase in encounter with GABA-IN's axo-shaft excitatory synapses could be due to the enlarged size of dendritic shafts or of PSD lengths. We verified that this was not a contributing factor, since the average diameter of the encountered and analyzed dendritic shafts were less for the 30mgKET group (Figure 3c).

3.2 | Higher dose of ketamine is associated with enhanced drebrin A labeling at excitatory synapses

The brains of animals that received 30mgKET dose of ketamine exhibited significantly enhanced proportion of GABA-IN's axo-shaft excitatory synapses labeled with drebrin A on the postsynaptic side compared to the 3mgKET group (Figure 4a) ($p = 0.0003$, $t = 4.858$, $df = 14$). The 30mgKET group also exhibited significantly enhanced proportion of PN's axo-spine excitatory synapses labeled with drebrin A on the postsynaptic side compared to the 3mgKET group (Figure 4b) ($p = 0.0013$, $t = 4.020$, $df = 14$). The 30mgKET group was also associated with significantly enhanced level of drebrin A labeling on the postsynaptic side of GABA-IN's axo-shaft excitatory synapses compared to the 3mgKET group (Figure 4c) ($p = 0.0007$, $t = 4.309$, $df = 14$). This reflects drebrin A labeling both at the plasma membrane and in the cytoplasm. The 30mgKET group was associated only

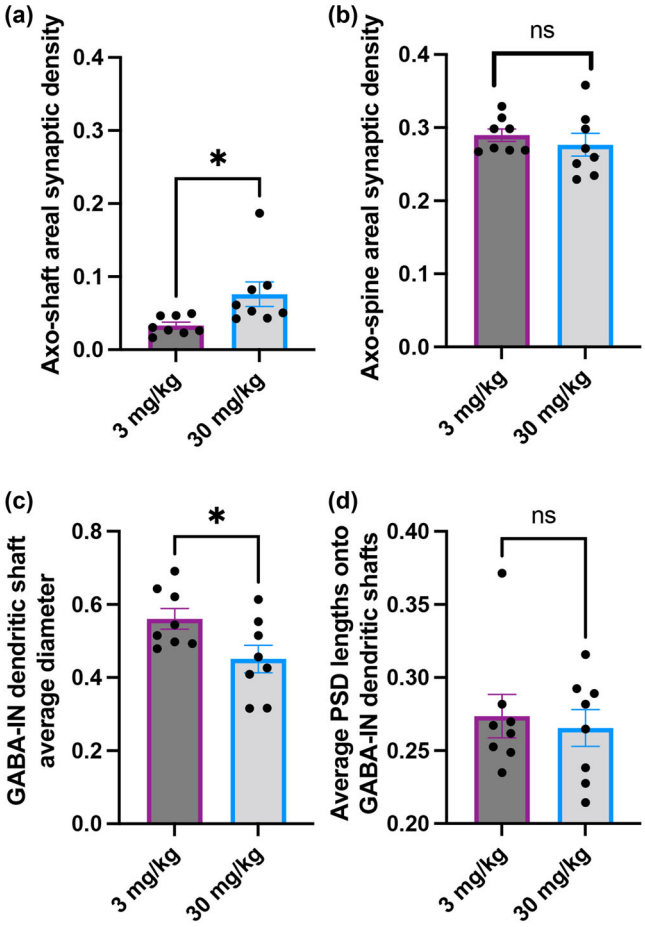

FIGURE 3 The dose of ketamine that enhanced resilience to ABA was associated with enhanced GABA-IN excitatory synaptic density. (a) GABA-IN's axo-shaft excitatory synaptic density is enhanced in the 30mgKET group compared to the 3mgKET group. $p = 0.0285$, $t = 2.442$, $df = 14$. (b) PN's axo-spinous excitatory synaptic density is not affected in the 30mgKET group compared to the 3mgKET group. $p = 0.4687$, $t = 0.7449$, $df = 14$. (c) Diameters of dendritic shafts belonging to GABA-IN is not increased by the 30mgKET dose of ketamine. $p = 0.0357$, $t = 2.324$, $df = 14$. (d) Postsynaptic density (PSD) length onto dendritic shafts belonging to GABA-IN is not increased by the 30mgKET dose of ketamine. $p = 0.6849$, $t = 0.4144$, $df = 14$. Bars represent mean ± SEM. *indicates $p < 0.05$, ns indicates 'not significant'. Abbreviations: ABA, activity-based anorexia; GABA-IN, GABAergic interneurons; PN, pyramidal neurons

with a trend of enhanced level of drebrin A labeling on the postsynaptic side of PN's axo-spine excitatory synapses compared to the 3mgKET group ($p = 0.0642$, $t = 2.009$, $df = 14$) (Figure 4d).

3.3 | Dose of ketamine that enhanced resilience to ABA through increased food consumption is associated with increased expression of drebrin A at the extrasynaptic location of axo-spinous excitatory synapses

The 30mgKET group exhibited an increased proportion of drebrin A labeling in the cytoplasm of the postsynaptic side of GABA-IN's axo-shaft synapses compared to the 3mgKET group (Figure 5c). Individuals with lower proportion of axo-shaft excitatory synapses labeled with drebrin A in the cytoplasm of the postsynaptic side exhibited increased food consumption during recovery after ABA2. This correlation between cytoplasmic drebrin A at axo-shaft synapses and food consumption was significant and negative (Figure 5d). The 30mgKET group exhibited an increased proportion of drebrin A labeling in the extrasynaptic location of the postsynaptic side of PN's axo-spinous synapses compared to the 3mgKET group (Figure 5a). Although this group difference did not reach statistical significance, correlation of this anatomical feature with a behavioral signature of ABA resilience, namely enhanced food consumption, was significant (Figure 5b). The correlation was positive, indicating that individuals with higher proportion of PN's axo-spinous excitatory synapses labeled with drebrin A at the plasma membrane of the postsynaptic side exhibited a greater increase in food consumption during recovery after ABA1.

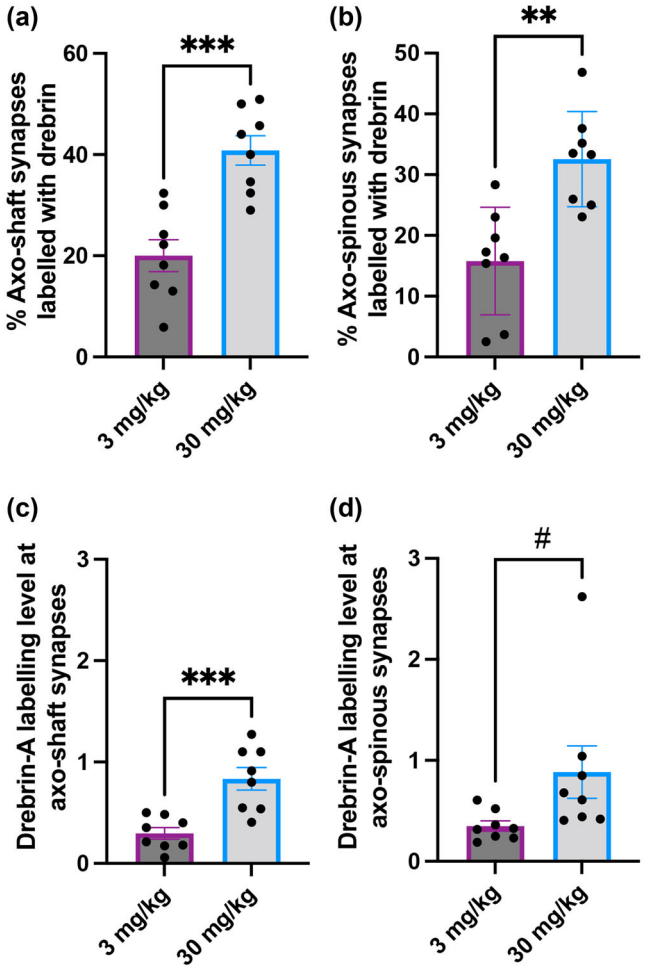

FIGURE 4 The higher dose of ketamine is associated with higher proportion and levels of drebrin A labeling at excitatory synapses. (a) The 30mgKET dose is associated with enhanced proportion of GABA-IN's axo-shaft excitatory synapses labeled with drebrin A on the postsynaptic side. $p = 0.0003$, $t = 4.858$, $df = 14$. (b) The 30mgKET dose is also associated with enhanced proportion of PN's axo-spinous excitatory synapses labeled with drebrin A on the postsynaptic side. $p = 0.0013$, $t = 4.020$, $df = 14$. (c) The 30mgKET dose is associated with enhanced drebrin A level on the postsynaptic side of GABA-IN's axo-shaft excitatory synapses significantly, compared to the 3mgKET group. $p = 0.0007$, $t = 4.309$, $df = 14$. (d) There is a trend toward higher drebrin A level for the 30mgKET group, relative to the 3mgKET group in on the postsynaptic side of PN's axo-spine excitatory synapses. $p = 0.0642$, $t = 2.009$, $df = 14$. Bars represent mean ± SEM. $^{\#}$indicates $0.05 < p < 0.1$, *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$. Abbreviation: GABA-IN, GABAergic interneurons

3.4 | Dose of ketamine promoting gain of resilience to ABA through decreased wheel activity correlated with the expression of drebrin A at the cytoplasmic location of axo-shaft excitatory synapses

The 30mgKET group exhibited an increased proportion of drebrin A labeling in the cytoplasmic location of the postsynaptic side of GABA-IN's axo-shaft excitatory synapses compared to the 3mgKET group (Figure 6a). Individuals with a lower proportion of GABA-IN's axo-shaft excitatory synapses labeled with drebrin A at the cytoplasmic location on the postsynaptic side exhibited a larger decrease in food-restriction-evoked wheel activity between ABA2 and ABA1. This correlation between cytoplasmic drebrin A at axo-shaft synapses and change in food-restriction-evoked wheel activity was significant and positive (Figure 6b).

3.5 | Dose of ketamine that enhanced resilience to ABA through weight restoration increased expression of drebrin A at the extrasynaptic location of GABA-IN's axo-shaft excitatory synapses

The 30mgKET group exhibited a higher proportion of GABA-IN's axo-shaft excitatory synapses labeled with drebrin A at the extrasynaptic location on the postsynaptic side compared to the 3mgKET group (Figure 7a). Individuals with higher proportion of axo-shaft synapses labeled with drebrin

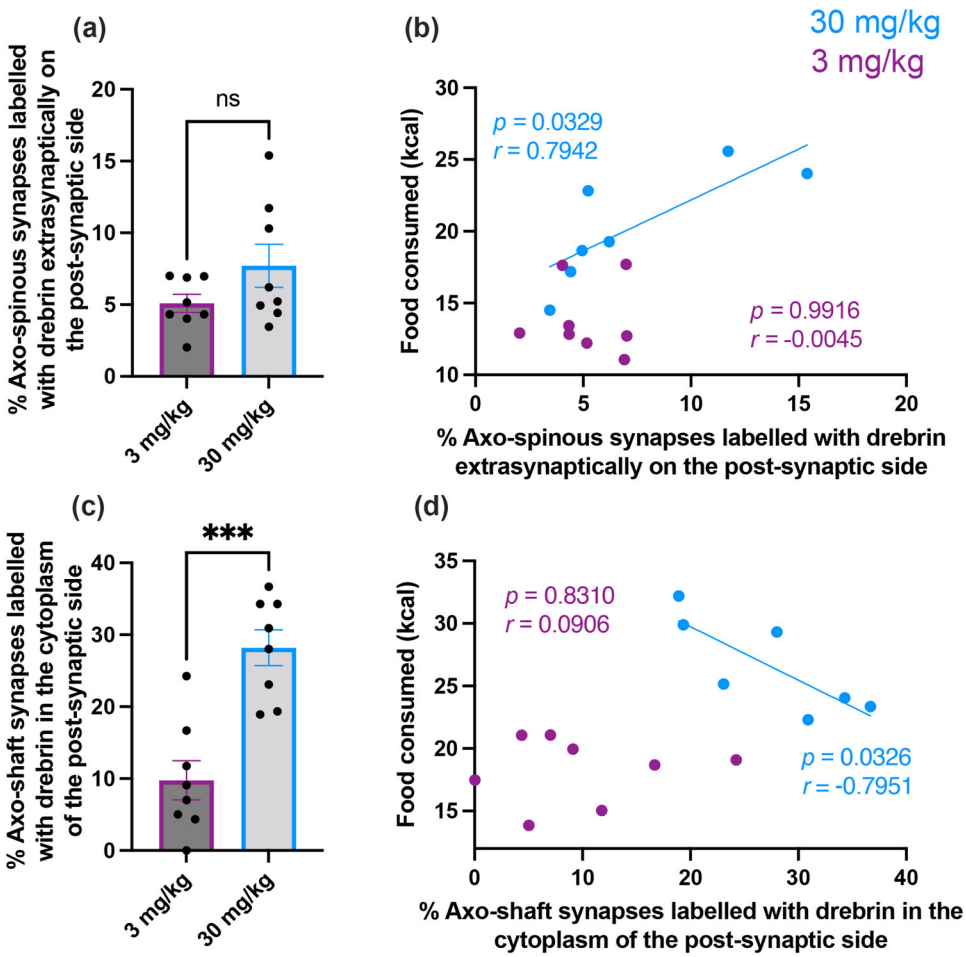

FIGURE 5 Individuals for whom the dose of ketamine enhanced resilience to ABA through increased food consumption exhibited increased expression of drebrin A at the extrasynaptic location of PN's axo-spine excitatory synapses. (a) Percentage of PN's axo-spinous excitatory synapses labeled with drebrin A extrasynaptically trended to be enhanced in the 30mgKET group compared to the 3mgKET group. $p = 0.1304$, $t = 1.607$, $df = 14$. (b) The 30mgKET group of animals with more PN's axo-spinous synapses labeled with drebrin A extrasynaptically on the postsynaptic side correlated significantly with food consumed during recovery post-ABA1. There was no correlation between PN's axo-spinous synapses labeled with drebrin A extrasynaptically on the postsynaptic side and food consumed during recovery post-ABA1 in the 3mgKET group. (c) Percentage of GABA-IN's axo-shaft excitatory synapses labeled with drebrin A in the cytoplasm was enhanced in the 30mgKET group compared to the 3mgKET group. $p = 0.0002$, $t = 4.999$, $df = 14$. (d) 30mgKET animals with less GABA-IN's axo-shaft synapses labeled with drebrin A cytoplasmically on the postsynaptic side increased food consumption during recovery post ABA2. Bars represent mean ± SEM. ***indicates $p < 0.001$, ns indicates 'not significant'. Abbreviations: ABA, activity-based anorexia; PN, pyramidal neurons

A in the extrasynaptic location of the postsynaptic side exhibited better maintenance of body weight compared to baseline (100%) during ABA2. This correlation between axo-shaft synapses labeled with drebrin A extrasynaptically on the postsynaptic side and body weight percentage during ABA2 was significant and positive (Figure 7b).

4 | DISCUSSION

4.1 | Summary

We analyzed drebrin A's expression pattern at excitatory synapses of the mPFC of adult ABA animals, searching for long-lasting signatures that underlie the protection provided by a single injection of ketamine 20 days earlier during mid-adolescence (Chen et al., 2018). We also sought to determine whether individual differences in behavioral responses to ketamine corresponded to individual differences in excitatory synapse density and drebrin A expression pattern. The new findings are that the efficacious 30mgKET dose and not the less efficacious dose of 3mgKET is associated with (1) increased number of excitatory synapses onto GABA-IN and (2) increased proportion of excitatory synapses with drebrin A of both

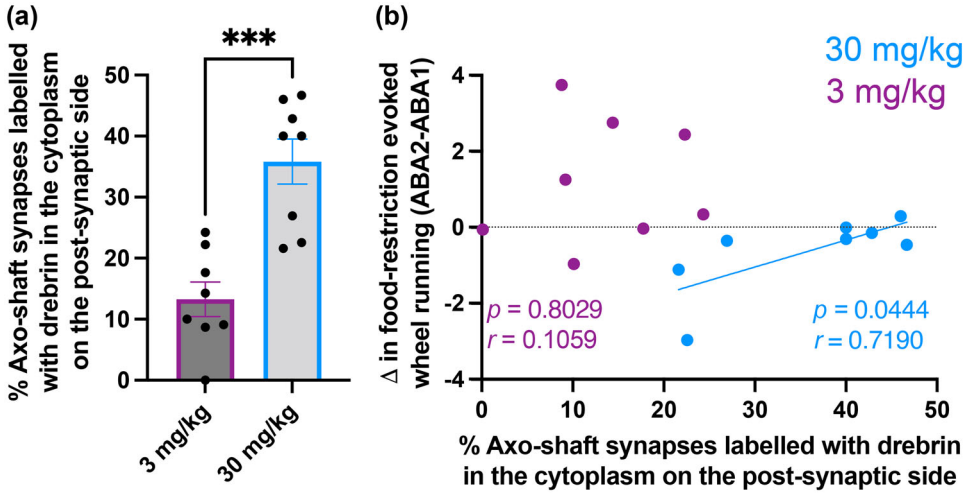

FIGURE 6 The dose of ketamine promoting gain of resilience to ABA through decreased wheel activity correlated with the expression of drebrin A at cytoplasmic location of GABA-IN's axo-shaft excitatory synapses. (a) Percentage of axo-shaft excitatory synapses labeled with drebrin A in the cytoplasm is enhanced in the 30mgKET group compared to the 3mgKET group. $p = 0.0003$, $t = 4.857$, $df = 14$. (b) The 30mgKET group of animals exhibiting less axo-shaft synapses with drebrin A in the cytoplasm of the postsynaptic side ran less in response to food restriction during ABA2, relative to ABA1. Negative *yy*-value indicates that the animal ran less in ABA2 than in ABA1. For the 3mgKET group, there was no correlation between axo-shaft synapses labeled with drebrin A in the cytoplasm and change in food-restriction evoked wheel activity between ABA1 and ABA2. Bars represent mean ± SEM. ***indicates $p < 0.001$. Abbreviations: ABA, activity-based anorexia; GABA-IN, GABAergic interneurons

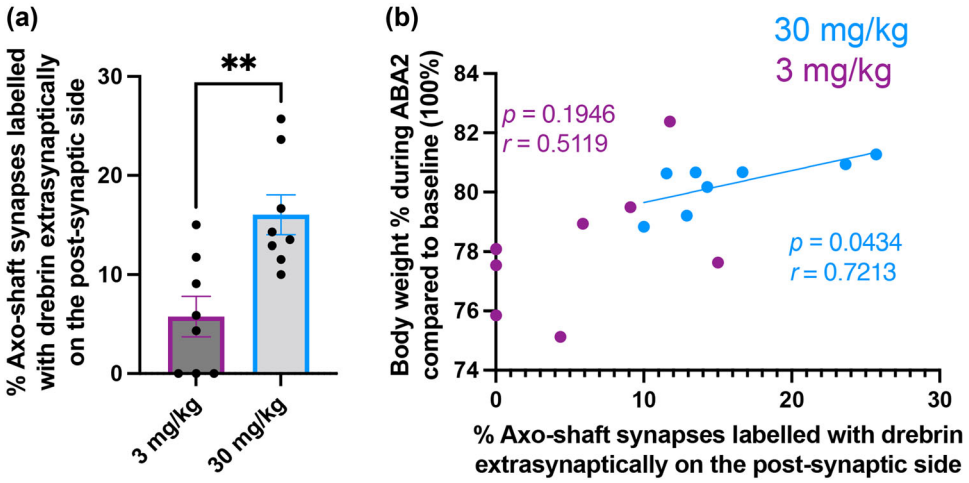

FIGURE 7 The dose of ketamine that enhanced resilience to ABA through weight restoration was associated with increased expression of drebrin A at the extrasynaptic location of GABA-IN's axo-shaft excitatory synapses. (a) Percentage of axo-shaft excitatory synapses labeled with drebrin A at the extrasynaptic location was enhanced in the 30mgKET group compared to the 3mgKET group. $p = 0.003$, $t = 3.578$, $df = 14$. (b) The 30mgKET group of animals with more axo-shaft synapses labeled extrasynaptically on the postsynaptic side correlated significantly with higher body weight percentage during ABA2 compared to baseline (100%). More axo-shaft synapses labeled extrasynaptically on the postsynaptic side were associated with protection against ABA. Bars represent mean ± SEM. **indicates $p < 0.01$. Abbreviations: ABA, activity-based anorexia; GABA-IN, GABAergic interneurons

GABA-IN and PN. Correlation analyses revealed that 30mgKET and not the 3mgKET dose (3) protects against ABA's maladaptive behaviors of excessive wheel running and voluntary food restriction, yielding better body weight retention (Chen et al., 2018) that may be through redistribution of drebrin A from the cytoplasm to extrasynaptic membranous sites of excitatory synapses of GABA-IN and PNs. These results are summarized in Figure 8. Additionally, correlation analyses revealed that 30mgKET and not the 3mgKET dose (4) evoked ABA resilience during ABA1, which is associated with a change in membranous drebrin A in PN but not in GABA-IN until after ABA2. We discuss each of these major findings below.

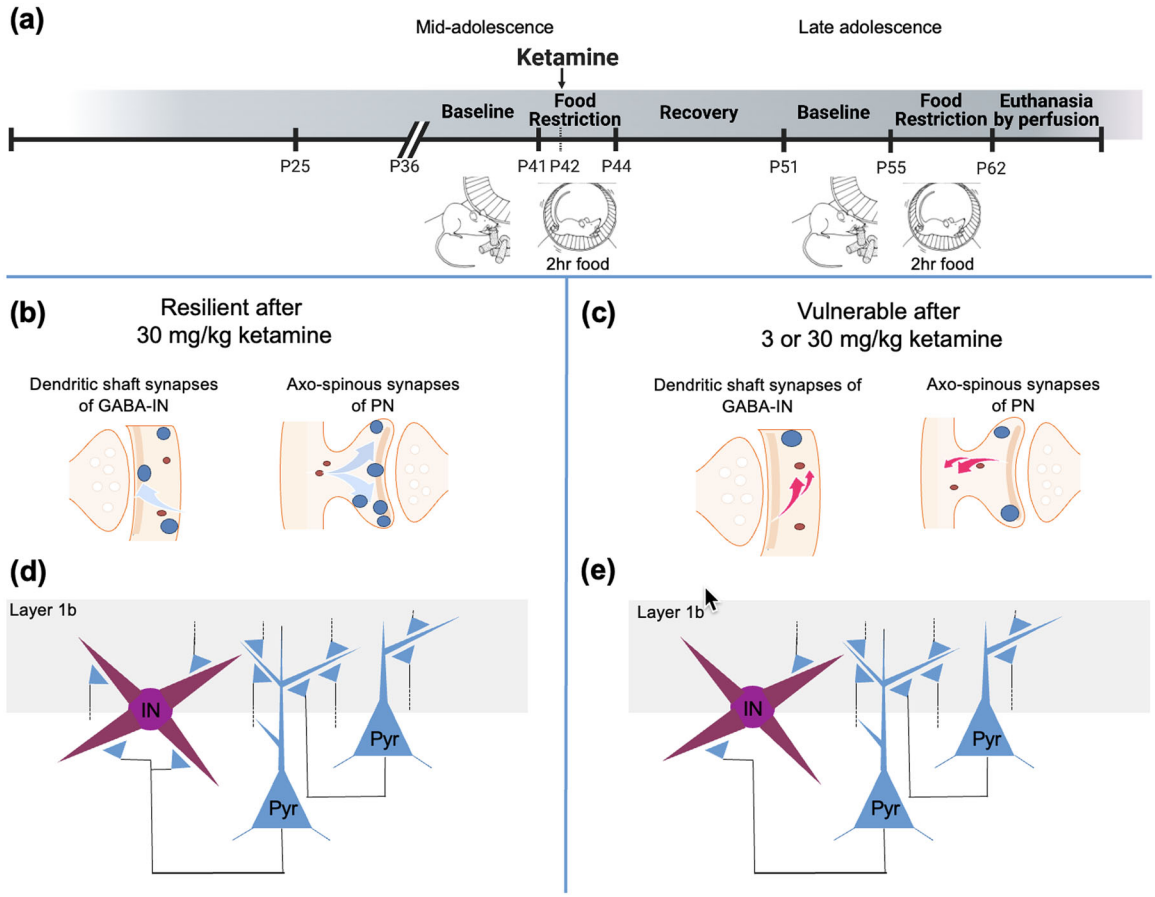

FIGURE 8 Graphical summary: Hypothesized molecular underpinning of individual differences in responsiveness to ketamine as it relates to the gain of ABA resilience. (a) Experimental design: Ketamine was injected into ABA mice during mid-adolescence. Brains were analyzed 20 days later, after animals had recovered from the second ABA. Panels B and C summarize intracellular differences in the excitatory synapses in mPFC layer 1b of animals that exhibited ABA resilience (b) versus ABA vulnerability (c) following ketamine treatment. The larger blue dots depict drebrin A located at the membrane, while smaller red dots depict drebrin A in the cytoplasm, away from the plasma membrane. Subjects which gained resilience to ABA after the 30mgKET ketamine injection exhibited enhanced drebrin A expression at GABA-IN's excitatory synapses on dendritic shafts and pyramidal neurons's axo-spinous excitatory synapses, particularly at membranous locations (panel B), where drebrin A may facilitate the activity-dependent insertions of glutamate receptor cargos into plasma membranes. Excitatory synapses of animals that remained vulnerable after 3 or 30mgKET of ketamine lacked enhancement of drebrin A. Where present, drebrin A labeling was at cytoplasmic locations near excitatory synapses on spines and shafts, rather than at plasma membranes (panel C), suggesting that drebrin A facilitated glutamate receptor cargos to be held as cytoplasmic reserve pools rather than promoting activity-dependent insertion of the cargos into plasma membranes. Panels D and E show sketch of the excitatory synaptic circuitry within layer 1b of subjects that gained resilience to ABA after the 30mgKET injection versus those that received 3mgKET. Those that gained resilience (panel D) exhibited enhanced excitatory synaptic input onto dendritic shafts of GABA-IN ("IN", magenta cells) but no change in excitatory synaptic input to pyramidal neurons ("pyr," blue cells) (panel D). Subjects that remained vulnerable to ABA after receiving either the 3 or 30mgKET dose of ketamine (Panel E) showed decreased number of excitatory inputs onto dendritic shafts belonging to GABA-IN. Panel A adapted from "Mouse Experimental Timeline", by BioRender.com (2022). Abbreviations: ABA, activity-based anorexia; GABA-IN, GABAergic interneurons; mPFC, medial prefrontal cortex

4.2 | An increase in the number of excitatory synapses onto GABA-IN is likely to include the VIP+ GABA-IN

Our ultrastructural analysis was restricted to layer 1b of mPFC. Here, we observed greater areal density of excitatory synapses onto GABA-IN of animals treated with 30mgKET, relative to those treated with 3mgKET (Figure 8b,c). It has been shown that knock-down of the GluN2B subunit of NMDAR subunit in GABA-INs of the mPFC occludes ketamine's antidepressant action, indicating the central role played by GABA-IN and of their expression of GluN2B subunits of NMDAR for ketamine's antidepressant effects (Gerhard et al., 2020). The increase of excitatory synapses onto GABA-IN of the ABA mice may have been triggered because ketamine targets and antagonizes excitatory synapses especially of GABA-IN, evoking those neurons to respond with increases in excitatory synapses—a form of homeostatic plasticity that brings neuronal excitability to the predrug setpoint (Turrigiano & Nelson, 2004). It is tempting to speculate that ketamine's ameliorative effects on ABA are dependent on the expression of GluN2B subunits in GABA-IN, as is suggested for ketamine's antidepressive actions.

Multiple excitatory afferents exist within layer 1: axon terminals of PFC layer 2/3 and layer 5 PNs, axons from amygdala (Little & Carter, 2013), as well as axons of mediodorsal thalamic nuclei and ventromedial thalamic nuclei (Anastasiades et al., 2020). Whether ketamine is efficacious because it can target all of these excitatory pathways or a subset of them is an important question for future studies. Among the multiple possible excitatory afferents, those arising from mediodorsal thalamic nuclei occur specifically in layer 1b of PFC, the region of interest in the current study, and primarily drives vasoactive intestinal peptide-positive GABA-INs (VIP+ GABA-IN) in the same layer (Anastasiades et al., 2020). Thus, the postsynaptic GABA-INs that are responsive to ketamine treatment are likely to include VIP+ GABA-IN that are driven by mediodorsal thalamic nuclei. These VIP+ GABA-IN inhibit L2/3 somatostatin+ GABA-IN, engaging a local disinhibitory circuit through suppression of dendritic inhibition of PN, allowing for synaptic plasticity across multiple layers beyond layer 1b (Anastasiades et al., 2020; Williams & Holtmaat, 2019). Thus, an increase of excitatory synapses in layer 1b GABA-IN following 30mgKET treatment may enhance synaptic plasticity of PN neurons in mPFC.

4.3 | Association of the efficacious dose of ketamine with increased proportion of excitatory synapses with drebrin A expression in both PN and GABA-IN 20 days later

Drebrin A is a neuron-specific F-actin-binding protein that occurs only on the postsynaptic side of excitatory synapses. Our rationale for studying drebrin A within ketamine-treated brains was to investigate whether drebrin A might mediate the ketamine-evoked homeostatic synaptic plasticity (Kavalali & Monteggia, 2020). It had been shown earlier that NMDAR blockade of layer 1 neuropil of cerebral cortex by the topical application of D-APV onto the cortical surface triggers trafficking of drebrin A, F-actin and GluN2A-NMDARs into dendritic spines within 30 min, lasting at least 2 h (Aoki et al., 2003; Fujisawa et al., 2006). Drebrin A knockout eliminates this D-APV-triggered increase of GluNA-NMDARs influx into spines (Aoki et al., 2009). We surmised that ketamine, a noncompetitive antagonist of NMDARs, might also trigger the influx of drebrin A into spines and modulate NMDARs trafficking into and within spines. Three new findings emerged from our study: (i) an efficacious dose of ketamine, like D-APV, is associated with increased levels of drebrin A's at excitatory synapses, suggesting that ketamine blockade of NMDARs evokes the trafficking of drebrin A toward excitatory synapses; (ii) this change is long-lasting, thereby being available to support synaptic changes during recovery from ABA1, during ABA2 and through recovery from ABA2; (iii) this change occurs not only in dendritic spines, as was reported previously following the D-APV treatment, but also at excitatory synapses formed onto GABA-INs (Figure 8). The functional significance of these changes is described below.

4.4 | Ketamine responders exhibit more membranous and less cytoplasmic drebrin A

Drebrin A possesses the property of inhibiting the actomyosin ATPase of the motor that translocates cytoplasmic cargos carrying glutamatergic receptors and other synapse-associated proteins along F-actin bundles near excitatory synapses. Because of this property, drebrin A in the cytoplasm may participate in keeping glutamate receptor cargos in the cytoplasmic reserve pool, while drebrin A at the plasma membrane may help to maintain glutamate receptor cargos near and at the plasma membrane (Aoki et al., 2017).

Individuals that responded well to 30mgKET by exhibiting the greatest food consumption showed higher levels of extrasynaptic membranous drebrin A in PN (Figures 5b and 8b, compared to Figure 5c). We believe that this location of drebrin A aids with the accumulation of glutamate receptor-containing cargos to the plasma membrane of dendritic spines. Strong behavioral correlations with drebrin A locations were found in GABA-IN as well: individuals that responded well to 30mgKET by exhibiting greater food consumption showed low levels of drebrin A in the cytoplasm (Figure 5d) after ABA1, while those with minimal body weight loss also showed greater levels of extrasynaptic membranous drebrin A later (after ABA2) (Figures 7b and 8b, compared to Figure 8c). We believe that these data are consistent with the idea that the efficacious dose of ketamine engages drebrin A in synaptic plasticity, yielding behavioral changes that contribute toward mitigating ABA vulnerability by aiding the trafficking of cargos carrying glutamate receptors from the cytoplasm to extrasynaptic membranous sites. Moreover, the difference in the timing of the membranous drebrin A across the two cell types (after ABA1 for PN, after ABA2 for GABA-IN) suggests that ketamine initially increases the E/I ratio in layer 1b of mPFC. This change in the E/I ratio may then evoke a longer-lasting change in INs that is to increase excitatory synaptic inputs to GABA-INs in layer 1b, returning the E/I ratio to the initial setpoint but through adjustments in the pathways controlling food intake (pyramidal neurons of the mPFC projecting to dorsal raphe (Du et al., 2022)) versus wheel running (pyramidal neurons of mPFC projecting to dorsal striatum (Santiago et al., 2021)), together yielding body weight control.

The mediodorsal thalamic nucleus-to-PFC excitatory pathway that is prevalent in layer 1b is proposed to be particularly important in ketamine's mechanism of action as an antidepressant (Miller et al., 2017). Genetic deletion of GluN2B-NMDAR subunit expressed by PN in the forebrain decreases depressive behavior and occludes ketamine's antidepressive action (Miller et al. 2014). Genetic deletion of GluN2B specifically in mPFC PNs results in enhanced excitatory synaptic drive from mediodorsal thalamic nucleus to mPFC through a postsynaptic mechanism, that is, increased miniature excitatory synaptic currents (mEPSCs), and reduces depression-like behavior (Miller et al., 2017). The increased mEPSCs in mPFC following the ketamine treatment, in turn, has been shown to be accompanied by increased expression of AMPA-type glutamate receptors and

BDNF through de-suppression of the mTOR protein synthesis pathway (Autry et al., 2011; Miller et al., 2014; Nosyreva et al., 2013; Zanos et al., 2016). The enhanced drebrin A expression at extrasynaptic membranous locations of dendritic spines belonging to PNs could also be supporting ketamine's antidepressive actions as well as amelioration of ABA.

4.5 | Future directions

Because we were investigating the changes evoked by ketamine long after it had cleared the body, the cellular and molecular basis for the acute effects of ketamine, such as increased food consumption and reduced anxiety behavior during ABA1 remains to be studied. Equally important is the need to know whether ketamine can be ameliorative for AN relapse during adulthood, not only among adolescents. Studies are underway examining this question.

This study focuses solely on ketamine's effect on glutamatergic synapses in mPFC layer 1b via drebrin A. How drebrin A might be affected by ketamine in other PFC layers and other brain regions remains to be explored. For example, analysis of layer 1a of mPFC revealed that excitatory synapse density on pyramidal neurons was reduced in the 30mgKET group, compared to the 3mgKET group, with no difference across the groups for GABA-IN synapses. Furthermore, drebrin A level at excitatory synapses in layer 1a was augmented by the 30mgKET dose for GABA-IN but not for PN (Li et al., unpublished observations; J. Li et al., 2022 SfN abstract). These differences in GABA-IN and pyramidal neuron synaptic densities seen in mPFC layer 1a compared to mPFC layer 1b could be due to ventromedial thalamic input onto neuron-derived neurotrophic factor positive (NDNF+) cells in layer 1a, whereas in layer 1b, the mediodorsal thalamus forms inputs onto vasoactive intestinal peptide positive (VIP+) cells in layer 1b (Anastasiades et al., 2020). These interneuron populations are involved in different inhibitory networks. VIP+ interneurons of layer 1b inhibit somatostatin-positive cells in layer 2/3, whereas NDNF+ interneurons of layer 1a inhibit parvalbumin positive cells and pyramidal cells in layer 2/3 (Anastasiades et al., 2020). There are various brain regions known to be implicated during induction of ABA in adolescence. In animals exhibiting vulnerability to ABA, excitatory and inhibitory tones in the hippocampus are altered. Excitatory tone is altered via enhanced expression of GluN2B NMDARs on PN, seen through immuno-labeling of GluN2B subunits and drebrin A (Chen et al., 2017). In animals exhibiting resilience to ABA through suppression of hyperactivity, enhanced expression of α4 and δ subunits of $GABA_A$ receptors at excitatory synapses of the PN in hippocampus was observed (Aoki et al., 2017, 2018, 2012) as well as enhanced GABA innervation onto PN in both hippocampus (Chen, Surgent, et al., 2016; Chen, Wable, et al., 2016; Aoki et al., 2018) and mPFC, specifically layer 5 (Chen et al., 2016). As for the ABA-induced increase in anxiety, which correlates strongly and positively with wheel running (Wable et al., 2015), knockdown of α4 subunits of $GABA_A$ receptors within nonpyramidal cells, including GABA-IN, of the hippocampus was shown to disrupt this relationship, suggesting a significant regulatory role of these neurons in the anxiety-evoked wheel running (Santiago & Aoki, 2022). Whether drebrin A modulates the location of a$\alpha\beta\delta$4-$GABA_A$ receptors at excitatory synapses is an interesting topic for future research. The pathway from PFC to dorsal striatum is known to generate hyperactivity, since chemogenetic activation of this pathway drives food restriction-evoked hyperactivity during ABA, while chemogenetic suppression of the pathway reduces food restriction-evoked hyperactivity (Santiago et al., 2021). The mPFC PNs projecting to dorsal raphe are also important in ABA vulnerability, with PFC GABAergic inhibition of these dorsal raphe-projecting PN underlying the reduced food consumption of ABA mice (Du et al., 2022). Altered $GABA_A$ receptor expression was also seen within the amygdala, where disinhibition via enhanced $GABA_A$ expression on GABA-IN correlated with enhanced ABA vulnerability (Wable et al., 2014). Thus, it is important to further investigate ketamine's mechanism of action through analysis of PFC, hippocampus, striatum, and amygdala, as well as pathways between these regions.

This study analyzed drebrin A, the adult isoform of the F-actin-binding protein, using an antibody that does not recognize drebrin E, the embryonic isoform. Our rationale for choosing to study drebrin A, and not drebrin E, is because genetic deletion of drebrin A results in the loss of activity-dependent trafficking of NMDARs (Aoki et al., 2017). Whether drebrin E, expressed by non-neuronal cells, also contributes to the protection of animals against ABA remains to be studied.

One technical limitation of our study is that we did not employ the disector method in calculating areal densities of synapses, so apparent increases in excitatory synapse density along GABA-IN dendrites (Figure 8b) could have been contributed by increases in the sizes of PSDs and shaft diameters of GABA-IN, which would have increased the probability of encounter with the enlarged profiles. However, our measurement of GABA-IN dendritic shaft diameters as well as PSD lengths showed no group difference. Therefore, the measured increase in areal density of excitatory synapses on GABA-IN most likely reflects increases in synapse number due to the ketamine treatment.

Studies are planned to co-label for drebrin A with specific NMDAR and AMPA receptor subunits, allowing us to further understand exactly which glutamatergic receptor subtypes are being affected by ketamine and drebrin A's ultrastructural distribution pattern. Chemogenetic modulation of the VIP+ IN projecting onto L2/3 somatostatin+ GABA-IN could further elucidate the importance of this specific pathway in ameliorating ABA vulnerability.

4.6 | Clinical relevance

One pressing question of clinical relevance is: Why different animals and individuals with AN respond differently to ketamine? Answers to this question may be applicable to another closely related question: Why is ketamine less efficacious to ABA mice in late adolescence (Aoki, 2020)? Ketamine antagonizes only those NMDARs that are activated, and the portion of the open NMDAR channels that are blocked is not likely to be 100% at the subanesthetic dose that we and others have used for correcting mood disorder-like behaviors (Zorumski et al., 2016). Thus, it may be that ketamine resets the E/I balance in mPFC by first blocking those excitatory synapses that are the most hyperactive during maladaptive behaviors. The animals of this study were injected with ketamine in the midst of food restriction-evoked hyperactivity and an hour away from the start of the feeding period (Chen et al., 2018). The sustained presence of drebrin A at the activated synapses combined with the transient temporal window to block those synapses supporting maladaptive behavior may enable individuals to switch mPFC's excitatory synaptic flow and behavior from maladaptive to adaptive types (exercise less and eat more), both of which are supported through synaptic circuitry in the mPFC. Based on this idea, the individual differences in responsiveness to ketamine may stem from differences in the behavioral states of the animal at the time of the injection. Additionally, the level of expression of NMDARs may vary across different cell types and individual difference may be especially large for an associational cortical area such as the mPFC that has the most protracted development pattern (Reh et al., 2020). These are some of the potentially contributing factors to individual differences in responsiveness to ketamine in ameliorating ABA vulnerability.

Pharmacotherapy treatment research for AN has been limited in providing clues for recovery and protection from relapse (Berends et al., 2018; Carter et al., 2004; Eddy et al., 2017; Khalsa et al., 2017). The disease decreases quality of life for those who experience it, as measured by Eating Disorder Quality of Life questionnaire, assessments of mental and physical health, and the Work and Social Adjustment Scale (Bamford & Sly, 2010). The severity and longevity of AN illness as well as its frequent co-morbidity with mood disorders, obsessive compulsive disorder, substance dependence, and generalized anxiety disorders also decrease quality of life (Bamford & Sly, 2010). AN is a dangerous illness, with starvation-induced heart failure as a leading cause of death among patients with AN (Giovinazzo et al., 2019). AN treatment is in dire need of improvement, and ketamine therapy is promising, helping to quickly alleviate symptoms and protect patients against relapse. Ketamine's therapeutic responses are thought to be due to circuit-level changes in the brain, lasting far after the drug has been cleared from the body (Keeler et al., 2021; Mkrtchian et al., 2021). Our study reveals that drebrin A plays some role in ketamine's long-lasting action (at least 20 days post ketamine injection) in mPFC in the ABA animals, perhaps through aid in circuit-level changes. This new finding is relevant for understanding ketamine's mechanism of action for treatment of AN, as well as its use as antianxiety and antidepressant (Gerhard et al., 2020).

Drug therapies such as ketamine, psilocybin, and MDMA have shown to be efficacious in transdiagnostic treatments for AN as well as major depressive disorder, obsessive compulsive disorder, generalized anxiety disorders, and substance-abuse disorders, (Mertens & Preller, 2021; Sarris et al., 2022) all of which are commonly comorbid with AN. Clinical studies using psilocybin for AN are currently recruiting or underway at institutions such as Imperial College London, Johns Hopkins University, and University of California San Diego. It is necessary to further study all of these drugs clinically and at a mechanistic level. They have shown to alleviate symptoms of mental illnesses quickly and with long-lasting efficacy, especially when compared to classically used psychiatric drugs such as Selective serotonin reuptake inhibitors (SSRIs) that often take months to elicit any response, which may be too slow for protecting patients with suicidal ideation (de Gregorio et al., 2018; Sarris et al., 2022). By further studying these drugs and the biological signatures they evoke, we get closer to making these therapeutic treatments more widely accepted and available, and closer to treating and healing more patients, saving many lives.

AUTHOR CONTRIBUTIONS

All authors significantly contributed to this research and approved of this manuscript.

ACKNOWLEDGMENTS

This study was supported by the Vulnerable Brain Project; New York University's Dean's Undergraduate Research Fund; New York University's Research Challenge Fund; Klarman Family Foundation Grant Program in Eating Disorders Research; Fulbright Scholarship; National Istitues of Health; National Science Foundation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All authors agree to sharing of raw and processed data, upon request.

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How to cite this article: Temizer, R., Chen, Y. -W., & Aoki, C. (2023). Individual differences in the positive outcome from adolescent ketamine treatment in a female mouse model of anorexia nervosa involve drebrin A at excitatory synapses of the medial prefrontal cortex. *Synapse*, *77*, e22253. https://doi.org/10.1002/syn.22253